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PRINCIPAL INVESTIGATOR: Edward P. Hasty, D.V.M.

CONTRACTING ORGANIZATION: The University of Texas Health Sciences Center at San Antonio
San Antonio, Texas 78229-3900

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Edward P. Hasty, D.V.M.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas Health Sciences Center
at San Antonio
San Antonio, Texas 78229-3900

E-Mail: HASTYE@UTHSCSA.EDU

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13. ABSTRACT (Maximum 200 Words)

Cancer is one of the leading causes of death in America and breast cancer is particularly threatening for women. In America 10% of women will be diagnosed with breast cancer resulting in the death of more than 40,000 of these women each year. Inheriting a single defect in genetic material causes about 5% of the cases of breast cancer. A gene that is commonly mutated in these inherited cases of breast cancer is called *BRCA2* (Breast Cancer susceptibility gene). *BRCA2* is called a tumor suppression gene because its function is essential for preventing cancer, as a result deletion of this function predisposes women to breast cancer. BRCA2 is important for repairing damage to genetic material, DNA, by virtue of its association to RAD51. The BRCA2-RAD51 complex repairs broken DNA and disruption of this interaction may predispose a woman to breast cancer. This work defines the Rad51-Brca2 interaction.

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Table of Contents

Introduction

BRCA2 is important for suppressing breast cancer. Women with one mutant copy of BRCA2 are predisposed to breast cancer with loss of heterozygosity being an important step in the oncogenic process. BRCA2 repairs DNA double-strand breaks by virtue of its association with Rad51 via homologous recombination. At the cellular level, disruption of recombination impedes proliferation and induces either senescence or apoptosis. In addition, BRCA2-deficient cells are sensitized to agents that generate DNA double-strand breaks and interstrand DNA crosslinks. Thus, therapeutic disruption of the BRCA2-RAD51 association should be efficacious in the treatment of cancer by acting as an adjuvant to radiation therapy and chemotherapy. Based on this reasoning we have developed a small peptide derived from a region encoded in exon 27 of BRCA2 that interacts with RAD51. Exposure to this peptide decreases proliferation and initiates apoptosis for cancer-derived cells grown in tissue culture. We proposed to characterize this peptide by determining its cytotoxic effect on a variety of cells derived from tumors that are both radiosensitive and radioresistant. Unfortunately, some of the batches of peptide are nonfunctional. Based on mass spectrometry racemization appears to affect function. Therefore, for the last year, we have focused on defining the Rad51-Brca2 exon 27 interaction by a mutagenic yeast two-hybrid assay. We have tested the ability of mutant Brca2 exon 27 to interact with Rad51 and have isolated numerous alterations that impair this interaction. In addition, we have tested Rad51's interaction with four proteins and have isolated 19 different Rad51 mutations that disrupt its ability to interact with some but not all of the proteins; thus, these are mutations that define specific protein-interacting domains. Currently we are developing Brca2 exon 27-mutant and Rad51-mutant cells designed to analyze these altered proteins. The ability of these altered proteins to rescue function will be tested in transgenic tissue culture cells exposed to a panel of genotoxic agents as previously described [1]. The sensitivity of Brca2 exon 27-deleted ES cells has been done. These cells are hypersensitive to cross-linking agents like mitomycin C (MMC) and cisplatin and to a topoisomerase 1 inhibitor, camptothecin. Rad51-mutant cells are currently being developed for this assay. These structure activity relationship analyses will better define the Rad51-Brca2 exon 27 interaction and which may prove useful for therapeutic development.

Body

RAD51 is important for repairing double-strand breaks in DNA by recombination [2]; interestingly, this function is likely to be essential since mammalian cells deleted for RAD51 exhibit chromosomal instability, are unable to sustain proliferation and senesce or die [3]. In order to function, RAD51 associates with BRCA2 [4], a protein important for suppression of breast cancer [5]. RAD51 associates with BRCA2 in two domains: the most amino - terminal domain is mediated by the BRC motifs encoded in exon 11 [6] and the most carboxy - terminal domain is mediated by a single region encoded in exon 27 [4]. COOH terminal deletions that remove some but not all of these regions increase replicative senescence and sensitivity to ionizing radiation and cross-linking agents, suggesting that the RAD51 - BRCA2 association is biologically important [7-9].

Currently, we are analyzing the Brca2 exon 27-Rad51 interaction from two different perspectives; both perspectives utilize yeast two hybrid. From the first perspective, the Rad51-interacting region of Brca2 exon 27 is defined by a mutation analysis of all amino acids. Each amino acid has been changed to alanine and these mutations were tested for their impact on interacting with Rad51. We found that multiple mutations either decreased or eliminated the

interaction with Rad51 by yeast two hybrid (not shown). From the second perspective, the Brca2 exon 27-interaction region of Rad51 is defined by a random mutagenesis screen as previously described by Dr. Lumir Krejci [10]. Dr Krejci, who is currently a postdoc in Dr. Patrick Sung's lab at Yale is our collaborator and he generated the mutant Gal 4 DNA binding domain - Rad51 cDNA (bait) during amplification in mutagenic bacteria. Our lab tested these randomly mutated baits for their interaction with Rad54, Rad51, the BRC motif, and Brca2 exon 27. We have isolated 18 mutations in Rad51 that impair its interaction with some but not all of these proteins (figure 1). Thus, these mutations will leave some interactions intact but impair others and could define the significance of these interactions.

Currently we are generating a tissue culture system to test the mutations in Brca2 exon 27. Previously we have generated mouse embryonic stem (ES) cells deleted for Brca2 exon 27 [7]. These mutant cells generate a protein with a C-terminal truncation that is impaired for homology direct repair [11]. These mutant cells exhibit genomic instability and hypesensitivity to γ-radiation, camptothecin and MMC [12]. We have modified this vector by exchanging the selection cassette with a cassette flanked by loxP sites as described in figure 2. By utilizing Cre-mediated recombination we will insert the wild type and altered forms of Brca2 exon 27 and then test for sensitivity to these agents and thus measure the impact the mutations have on function and the relative importance of these amino acids for their interaction with Rad51. The modified vector is made and has been electroporated into ES cells.

We are also generating a tissue culture system to test the mutations in Rad51. We have constructed a targeting vector with a selection cassette with loxP sites that will be used to integrate in the different Rad51 cDNAs as described in figure 3. Generation of these cells is complicated since deletion of Rad51 is a cell lethal [3]. Thus, the first step is to introduce a Rad51 cDNA controlled by a doxycycline-inducible system. Then the second copy of Rad51 will be targeted to integrate the altered Rad51 cDNAs. These cells will be used to define the function of the altered cDNAs. At this time we have targeted the first copy of Rad51 and have performed the first Cre-mediated step as shown in figure 3. Currently we are introducing the tTA adjacent to the Rad51 promoter.

Key Research Accomplishments

1) Defined the Rad51-interacting domain in Brca2 exon 27.
2) Better defined the Brca2 exon 27-interacting domain in Rad51.
3) Generated a targeting vector that will introduce loxP sites to Brca2.
4) Generated a targeting vector that will introduce loxP sites to Rad51.
5) Targeted the first copy of Rad51.
6) Performed the first Cre-mediated recombination step.

Reportable Outcomes

Publication: Donoho G, Brenneman MA, Cui TX, Donoviel D, Vogel H, Goodwin EH, Chen DJ, Hasty P. Deletion of Brca2 exon 27 causes hypersensitivity to DNA crosslinks chromosomal instability, and reduced life span in mice. Genes Chromosomes Cancer. 2003, 36:317-31.

Publication: Marple, T., Li, H. & Hasty, P. A genotoxic screen: rapid analysis of cellular dose-response to a wide range of agents that either damage DNA or alter genome maintenance pathways. *Mutat Res* **554**, 253-266 (2004).

Publication: Hasty P. The impact of DNA damage, genetic mutation and cellular responses on cancer prevention, longevity and aging: observations in humans and mice. Mech Ageing Dev. 2005, 126(1):71-7.

Patent No.: 6,037,125
Title: Disruption of the Mammalian Rad51 Protein & Disruption of Proteins that Associate with Mammalian Rad51.

Patent No.: 6,057,104
Title: Disruption of the Mammalian Rad51 Protein & Disruption of Proteins that Associate with Mammalian Rad51 for Hindering Cell Proliferation

Conclusions

We are investigating the Rad51-Brca2 exon 27 interacting regions. We have analyzed all the amino acids within the minimal region Brca2 exon 27 needed for the Rad51-interaction and have found many, but not all amino acids to be important. We have also isolated 19 mutations in Rad51 that impair its interaction with some but not all its partners; including Brca2 exon 27. We are currently developing a tissue culture system to test these mutations in both Brca2 exon 27 and Rad51.

Figures

Class	MT	Brca2 Ex27	Rad54	mRad51	BRC
I	M1-2	O	O	O	W
	M1-4	O	O	O	W
	4M-8	O	O	O	X
	4M-14	O	O	O	X
II	M1-1	O	O	W	O
	M1-3	O	O	W	O
	4M-26	O	O	W or X	O
III	2M-4	X	O	O	O
IV	2M-1	X	X	X	O
	2M-2	X	X	X	O
	4M-9	X	X	X	O
	4M-23	VW	VW	X	O
	4M-19	X or W	X	X	O
	4M-31	X	X	X	O
V	2M-3	W	O	W	X
	4M-1	W	O	W	W
	4M-10	W	O	W	X
	4M-20	X	O	X	X
	4M-27	W	O	W	W
	4M-30	X	O	X	X
VI	4M-2	W	O	W	O
	4M-29	W or X	O	X	O

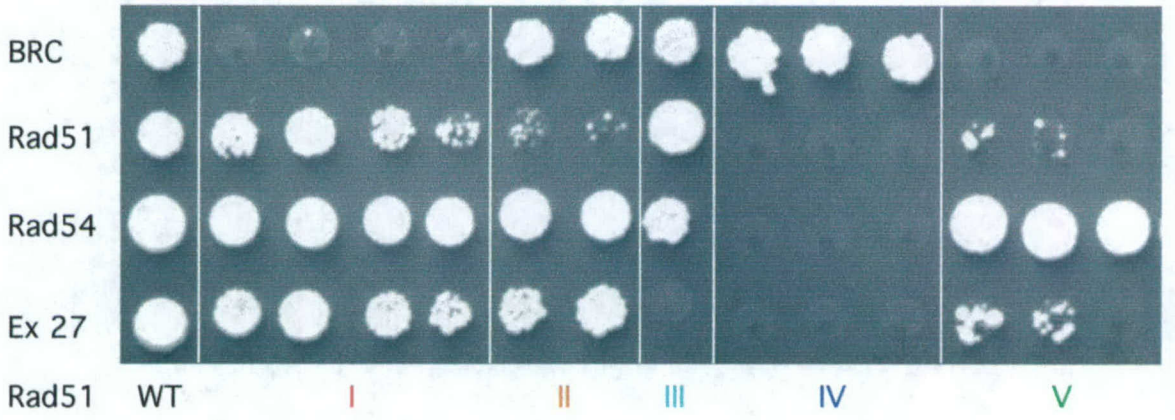

Figure 1. Definition of Rad51's interaction with Brca2 exon 27 and other proteins. No effect, O; weak interaction, W; no interaction, X. The mutations are loosely classified based on their interactions.

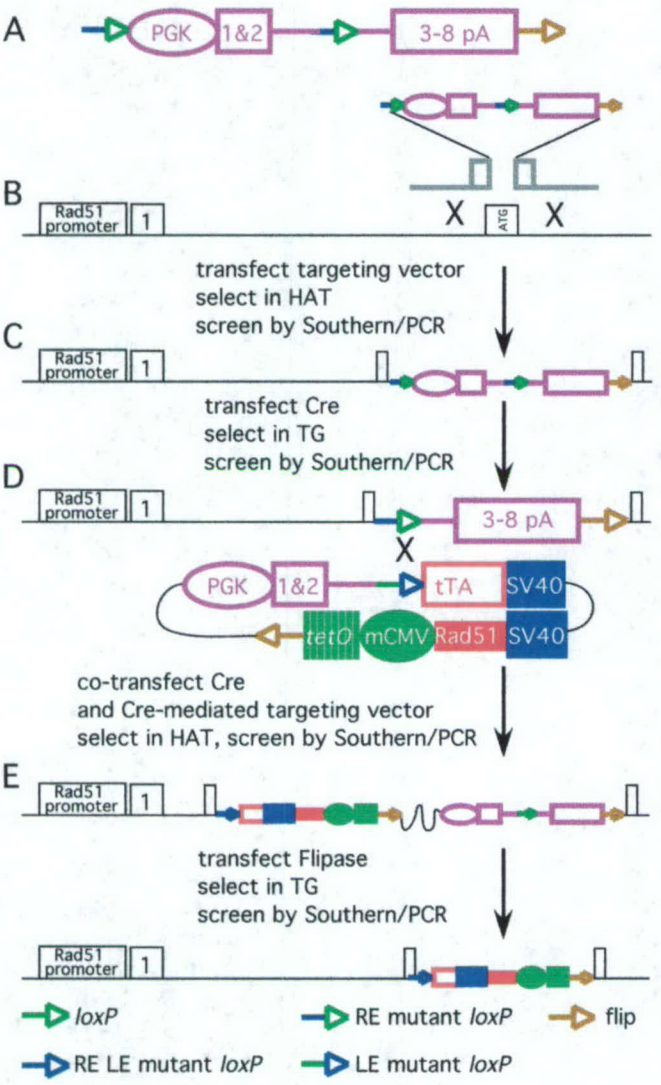

Figure 2. Analysis of Brca2 exon 27 variants by high throughput exon replacement. **A**. The *Hprt* minigene (purple) has a phosphoglycerate kinase 1 (PGK) promoter [13], exons 1 and 2 (box labeled 1&2), intron (straight line) and exons 3-8 with polyadenylation sequences (box labeled 3-8 pA). A RE mutant *loxP* (blue-green arrow) is 5' to PGK and another RE mutant *loxP* is in the intron. A flip site (orange arrow) is 3' to the *Hprt* minigene. **B**. Placement of the RE mutant *loxP* site at the position of Brca2 exon 27 by gene targeting. The *Hprt* minigene is flanked by sequences (gray line) homologous to the genomic target (straight thin black line) in the second exon such that exon 27 and some surrounding sequence is deleted. Cells are selected in HAT and screened by Southern analysis or PCR for homologous recombination. **C**. Excision of one RE mutant *loxP*, the PGK promoter and *Hprt* exons 1 and 2 after transient transfection with Cre recombinase (select in TG). One RE mutant *loxP*, the flip site and *Hprt* exons 3-8 remain. **D**. Integration of the Cre-mediated targeting vector with Brca2 exon 27 variant (*). This vector has the 5' half of the *Hprt* minigene, LE mutant *loxP* (green-blue arrow), flip and plasmid backbone (wavy line). Cre-mediated recombination results in restoration of the Hprt minigene and is selected in HAT. An RE LE mutant *loxP* (blue arrow) and a *loxP* (green arrow) are formed. **E**. Flip-mediated excision of *Hprt* minigene and *loxP* (optional). Removes *Hprt* minigene, plasmid backbone, *loxP* and one flip. The transgene remains next to the promoter. Select in TG.

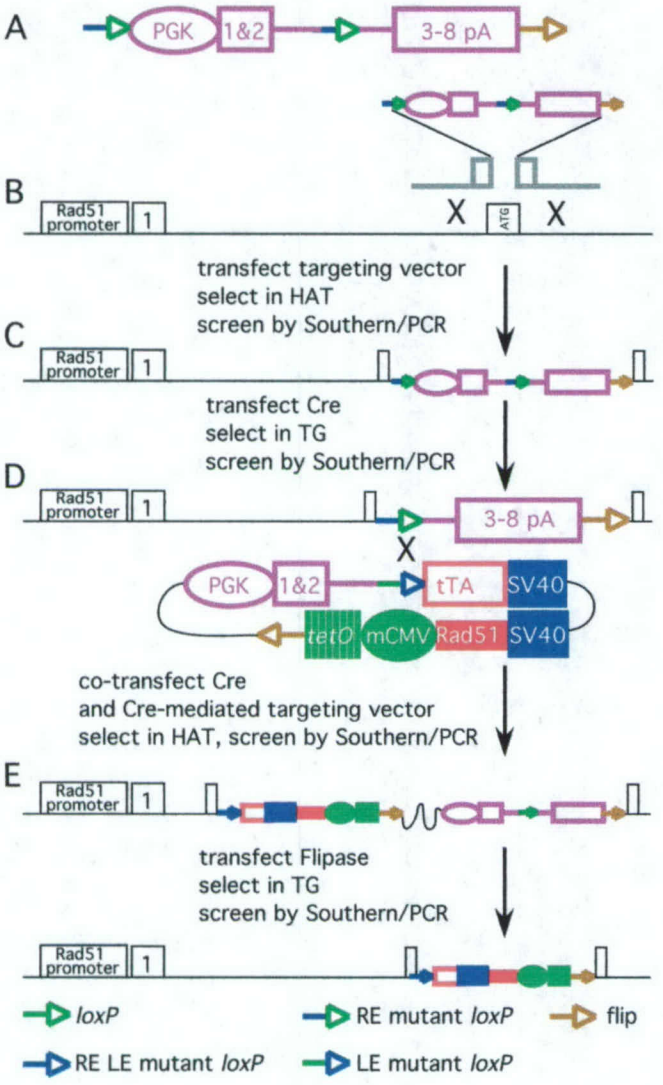

Figure 3. Analysis of Rad51 variants by high throughput knockin.

A. The *Hprt* minigene: same as described for figure 2.

B. Placement of the RE mutant *loxP* site adjacent to the endogenous Rad51 promoter by gene targeting: done as described for figure 2.

C. Excision of the 5' half of the Hprt minigene as described for figure 2.

D. Integration of the Cre-mediated targeting vector needed for inducible expression of wild-type Rad51. This vector has a tTA with a Kozak ATG (red open rectangle) and a Rad51 cDNA (red box) expressed by the minimal CMV promoter with tetO sequences (green oval and green rectangels), the 5' half of the *Hprt* minigene, LE mutant *loxP* (green-blue arrow), flip and plasmid backbone. Thus, the tTA is expressed by the Rad51 promoter and the Rad51 cDNA is controlled by the tTA which can be regulated with doxycycline. Selection is the same as for figure 2.

E. Flip-mediated excision of *Hprt* minigene and *loxP* (optional): same as for figure 2.

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Appendices

1) **Listing of Personnel supported by this research grant (see attached)**
2) **Meeting Abstracts (see attached)**
3) **Publications (see attached)**

APPENDIX ITEM #1 -- Personnel supported by grant:

1) E. Paul Hasty, D.V.M.
2) Valerie Boka
3) Shengmei Ma

APPENDIX ITEM #2 – Meeting Abstracts:

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APPENDIX ITEM #3 – Publications:

Publication: Donoho G, Brenneman MA, Cui TX, Donoviel D, Vogel H, Goodwin EH, Chen DJ, Hasty P. Deletion of Brca2 exon 27 causes hypersensitivity to DNA crosslinks chromosomal instability, and reduced life span in mice. Genes Chromosomes Cancer. 2003, 36:317-31.

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Publication: Hasty P. The impact of DNA damage, genetic mutation and cellular responses on cancer prevention, longevity and aging: observations in humans and mice. Mech Ageing Dev. 2005, 126(1):71-7.

GENES, CHROMOSOMES & CANCER 36:317–331 (2003)

RESEARCH ARTICLE

Deletion of *Brca2* Exon 27 Causes Hypersensitivity to DNA Crosslinks, Chromosomal Instability, and Reduced Life Span in Mice

Greg Donoho,[1] Mark A. Brenneman,[2] Tracy X. Cui,[3] Dorit Donoviel,[1] Hannes Vogel,[4] Edwin H. Goodwin,[3] David J. Chen,[5]* and Paul Hasty[1]*

[1]Lexicon Genetics, Inc., The Woodlands, Texas
[2]Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, New Mexico
[3]Biosciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico
[4]Department of Pathology, Baylor College of Medicine, Houston, Texas
[5]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California

The *Brca2* tumor-suppressor gene contributes to genomic stability, at least in part by a role in homologous recombinational repair. BRCA2 protein is presumed to function in homologous recombination through interactions with RAD51. Both exons 11 and 27 of *Brca2* code for domains that interact with RAD51; exon 11 encodes eight BRC motifs, whereas exon 27 encodes a single, distinct interaction domain. Deletion of all RAD51-interacting domains causes embryonic lethality in mice. A less severe phenotype is seen with BRAC2 truncations that preserve some, but not all, of the BRC motifs. These mice can survive beyond weaning, but are runted and infertile, and die very young from cancer. Cells from such mice show hypersensitivity to some genotoxic agents and chromosomal instability. Here, we have analyzed mice and cells with a deletion of only the RAD51-interacting region encoded by exon 27. Mice homozygous for this mutation (called *brca2*^{lex1}) have a shorter life span than that of control littermates, possibly because of early onsets of cancer and sepsis. No other phenotype was observed in these animals; therefore, the *brca2*^{lex1} mutation is less severe than truncations that delete some BRC motifs. However, at the cellular level, the *brca2*^{lex1} mutation causes reduced viability, hypersensitivity to the DNA interstrand crosslinking agent mitomycin C, and gross chromosomal instability, much like more severe truncations. Thus, the extreme carboxy-terminal region encoded by exon 27 is important for BRCA2 function, probably because it is required for a fully functional interaction between BRCA2 and RAD51.

INTRODUCTION

BRCA2 is a major breast cancer susceptibility gene in humans (Wooster et al., 1995). Women with a heterozygous mutation of *BRCA2* are predisposed to breast cancer because of the loss of heterozygosity. Because loss of heterozygosity usually accompanies tumor formation, *BRCA2* has been defined as a tumor-suppressor gene. There are two basic categories of tumor-suppressor proteins: gatekeepers and caretakers (Kinzler and Vogelstein, 1997). Gatekeepers regulate the cell cycle and cell death; well-known examples are p53 and pRB (retinoblastoma). Caretakers repair the genome; examples include some of the mismatch repair and nucleotide excision repair proteins. BRCA2 appears to fit into both categories because it is important for the repair of damaged DNA (Sharan et al., 1997; Morimatsu et al., 1998; Moynahan et al., 2001b; Tutt et al., 2001; Xia et al., 2001) and is also involved in control of the G2/M cell cycle checkpoint (Chen et al., 1999).

A role for the BRCA2 protein in the repair of DNA double-strand breaks by homologous recombinational repair (HRR) was first proposed because of its association with the recombinational strand transfer protein RAD51 in mice and humans (Mizuta et al., 1997; Sharan et al., 1997; Wong et al., 1997; Chen J et al., 1998; Chen PL et al., 1998; Katagiri et al., 1998; Marmorstein et al., 1998). Both

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G. Donoho and M. Brenneman contributed equally to this work.

G. Donoho is currently at Department of Mouse Genomics, Pharmacia Corporation, 301 Henrietta Street, Kalamazoo, MI 49007.

T. Cui is currently at Department of Physiology, University of Michigan, Med. Sci. II 6815, Ann Arbor, MI 48109.

*Correspondence to: Dr. Paul Hasty, Department of Molecular Medicine and Institute of Biotechnology, University of Texas Health Science Center, San Antonio, TX 78245-3207.
E-mail: hastyc@uthscsa.edu. Correspondence may also be sent to Dr. David J. Chen, Life Sciences Division, Lawrence Berkeley National Laboratory, MS 74-157, 1 Cyclotron Road, Berkeley, CA 94720. E-mail: DJChen@lbl.gov.

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proteins are important for protecting cells against ionizing radiation damage, the most lethal component of which is DNA double-strand breaks (Ritter et al., 1977.). Two regions of BRCA2 associate with RAD51. A central region has eight repeats of a domain called the BRC motif, encoded in exon 11 (Wong et al., 1997; Chen PL et al., 1998; Katagiri et al., 1998; Sarkisian et al., 2001). The other region is a single domain in the extreme carboxy-terminus, distinct from the BRC motifs, encoded in exon 27 (Mizuta et al., 1997; Sharan et al., 1997). The importance of the BRCA2–RAD51 interaction is supported by data from mice with truncations of BRCA2 that remove RAD51-interaction domains. Truncations that omit all of the BRC motifs and the exon 27 domain result in embryonic lethality, decreased cellular proliferation, and hypersensitivity to ionizing radiation (Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). These *brca2* nullizygous mice are phenocopies of *rad51* nullizygous mice (Lim and Hasty, 1996). Shorter truncations in BRCA2 that remove the exon 27 domain and some, but not all, of the BRC motifs result in partial embryonic lethality, sterility, and increased incidence of cancer in mice, together with cellular phenotypes of decreased proliferation, hypersensitivity to DNA damage, and genomic instability (Connor et al., 1997; Friedman et al., 1998; Patel et al., 1998; Tutt et al., 1999; Yu et al., 2000). These phenotypes suggest that recombinational repair is impaired by these mutations, but not completely ablated, such that cells and mice can survive.

Previously, we reported mouse embryonic stem cells (ES cells) and fibroblasts with a short carboxy-terminal deletion that removes only the region encoded by exon 27 (all of the BRC motifs are intact). These *Brca2*-mutant cells undergo premature replicative senescence and are hypersensitive to γ radiation (Morimatsu et al., 1998). Recently, they have been shown to be deficient in HRR of a specifically induced chromosomal break (Moynahan et al., 2001b). This implies that the single RAD51-binding domain in exon 27 is important for fully efficient recombinational repair.

Here we report on the phenotype of mice and mouse embryonic fibroblasts (MEFs) with the exon 27 deletion (designated $brca2^{lex1}$). Compared to control mice, the $brca2^{lex1}$ mice exhibit a decreased life span that may be partially attributable to early onsets of cancer and sepsis. However, the $brca2^{lex1}$ allele exhibits a normal Mendelian pattern of inheritance, indicating no loss of viability in homozygous embryos. In addition, $brca2^{lex1}$ mice are of normal size and are fertile, unlike mice with the larger carboxy-terminal truncations that omit some of the BRC motifs. Thus, the $brca2^{lex1}$ mutation is very mild and suggests only a modest impairment of BRCA2 activity. However, even though $brca2^{lex1}$ mice exhibit a mild phenotype, MEF cells bearing this mutation exhibit impaired growth, gross chromosomal instability, and severe hypersensitivity to the interstrand crosslinking agent mitomycin C, much like cells with the more severe truncations that omit some BRC repeats.

MATERIALS AND METHODS

Histologic Analysis

Histologic sections were taken from tissues or tumors fixed in 4% buffered formalin, embedded in paraffin blocks, cut into 4-μm sections, and stained according to standard procedures.

Mouse Embryonic Fibroblast Cultures

Primary and immortalized MEF cells were grown in Minimum Essential Medium-α (GibcoBRL, Rockville, MD) with 10% fetal bovine serum (Nova-Tech, Grand Island, NE). Cultures were maintained at 37°C in a humidified incubator with 6% carbon dioxide. For passage of primary MEF cells, confluent cultures were trypsinized and replated with an inoculum of 1×10^5 cells per 10-cm dish.

γ Radiation Exposures

Wild-type and $brca2^{lex1/lex2}$ immortalized MEFs were plated for colony-formation assays at 500 cells/10-cm dish and 1000 cells/10-cm dish, respectively. Triplicate cultures were plated for each radiation dose to be tested. Twelve hours after plating, cells were irradiated by use of a Mark I model 68A High Dose Rate ^{137}Cesium Source Chamber Irradiator (J.L. Sheppard and Associates, Freemont, CA), then returned to incubation at 37°C. Eleven days after plating, cultures were fixed and stained with 0.2% Crystal Violet in ethanol. Colonies of 50 or more cells were counted.

Mitomycin C Exposures

Immortalized wild-type and $brca2^{lex1/lex2}$ MEFs were plated for colony-formation assays at 500 cells/10-cm dish and 1000 cells/10-cm dish, respectively, with mitomycin C (Sigma, St. Louis, MO) at final concentrations of 0 M, 3×10^{-9} M, 1×10^{-8} M, 3×10^{-8} M, 1×10^{-7} M, and 3×10^{-7} M. Triplicate cultures were plated for each mitomycin C dose to be tested. After 11 days, cultures were

stained with 0.2% Crystal Violet in ethanol. Colonies of 50 or more cells were counted.

Chromosome Slide Preparation

For chromosome harvest, Colcemid (Gibco-BRL) was added to subconfluent MEF cultures to a final concentration of 0.05 μg/ml for 1.5 to 2 hr. The cells were then trypsinized, centrifuged, and resuspended in hypotonic saline (0.075 M KCl) at 37°C for 12 min. The cells were fixed in a 3:1 mix of methanol and acetic acid and stored at −20°C. Fixed cell suspensions were dropped onto wet glass slides and allowed to air-dry. For analysis of chromosome aberrations, slides were Giemsa-stained.

Fluorescence In Situ Hybridization

After 2 weeks of aging in air, chromosome slides were denatured in 70% formamide in 2× SSC at 70°C for 2 min. A synthetic single-strand telomere-repeat oligomer, $(TTAGGG)_7$, was labeled with Oregon green-dCTP (Molecular Probes, Eugene, OR) by use of a terminal transferase reaction kit (Roche Applied Science, Indianapolis, IN). A probe for the centromere-proximal mouse major satellite repeat (TGG AAT ATG GCG AGA AAA CTG AAA ATC ATG GAA AAT GAG A) was labeled by the same method with Cy3-dCTP (Amersham Life Science, Piscataway, NJ). A hybridization mixture consisting of 0.25 μg/ml telomere probe DNA and 0.25 μg/ml centromeric probe DNA in 50% formamide, 2× SSC, was applied to the slides. After overnight hybridization at 37°C, the slides were washed in 2× SSC at 42°C three times, 15 min each, and then for 5 min with 1% Triton-X 100 in phosphate buffer (pH 8.0). Chromosomes were counterstained with DAPI in antifade solution.

RESULTS

Breeding of *brca2^{lex1}* and *brca2^{lex2}* Mice

Previously, we developed mouse ES and MEF cells with a compound heterozygous mutation for *Brca2* in an isogenic 129SvEv background (Morimatsu et al., 1998). The two mutated alleles are designated *brca2^{lex1}* and *brca2^{lex2}*. The *brca2^{lex1}* mutation is a precise deletion of exon 27, whereas the *brca2^{lex2}* mutation is a deletion of exon 27 and part of exon 26. The impact of these mutations was observed in mice generated from the ES cells. The mice in this study were in a 129SvEv–C57Bl6 crossbred background (i.e., derived from founding C57Bl6 X 129SvEv F1 mice).

The progeny from *brca2^{lex2}* heterozygous breeding pairs were observed. Of 56 mice genotyped from *Brca2$^{+/lex2}$* crosses, there were 32 *Brca2$^{+/lex2}$* and 24 *Brca2$^{+/+}$* mice. No *brca2$^{lex2/lex2}$* mice were generated from *Brca2$^{+/lex2}$* breeding pairs, demonstrating that the *brca2^{lex2}* mutation is an embryonic lethal. We also genotyped day 10.5 embryos from a *Brca2$^{+/lex2}$* female crossed with a *brca2$^{lex1/lex2}$* male. In addition to *Brca2$^{+/lex2}$* and *brca2$^{lex1/lex2}$* embryos, which were normal in appearance, one *brca2$^{lex2/lex2}$* embryo was recovered, which was partially resorbed and appeared to have arrested at approximately embryonic day 6 (Fig. 1A). This is a similarly severe phenotype, as observed with truncations that delete all of the identified RAD51-interaction domains (Ludwig et al., 1997; Sharan et al., 1997; Suzuki et al., 1997). These data imply that the *brca2^{lex2}* allele is functionally null, or at least close to null, because, if the *brca2^{lex2}* allele produced a stable protein, then the *brca2$^{lex2/lex2}$* mice and cells should be viable, much like the mice and cells that are deleted for some of the BRC motifs or like the *brca2$^{lex1/lex1}$* mice and cells. It is unlikely that the *brca2^{lex2}* allele produces a dominant-negative protein that causes the phenotype observed in *brca2$^{lex1/lex2}$* cells. If this were the case, then a mutant phenotype should also be observed in *Brca2$^{+/lex2}$* cells, but it was not. The *brca2^{lex2}* mutation likely destabilizes the transcript, given that a corresponding mRNA could not be detected by RT-PCR from *brca2$^{lex1/lex2}$* cells, although a transcript corresponding to the *brca2^{lex1}* allele was readily detected (data not shown).

Given that the *brca2^{lex2}* allele is an early embryonic lethal, the *brca2^{lex1}* allele must produce a partially functional protein because *brca2$^{lex1/lex2}$* cells and mice are viable. The *brca2^{lex1}* mutation allows transcription because exon 27 was replaced with a splice acceptor, stop codon, and polyadenylation site (part of the *Hprt* minigene positive selection cassette) (Morimatsu et al., 1998). The predicted fusion of *Brca2* exon 26 into the new splice acceptor was demonstrated by Northern blot and RT-PCR from *brca2$^{lex1/lex2}$* cells, and yields a transcript in which the nucleotides that code for amino acids 3140–3328 are deleted (Morimatsu et al., 1998). Further analysis has shown that this transcript is translated to a truncated protein, which retains the ability to co-immunoprecipitate with RAD51 from nuclear extracts, presumably through the BRC motifs (Moynahan et al., 2001b). Thus, the *brca2^{lex1}* allele produces a protein that contains more than 94% of the wild-type amino acid sequence (includ-

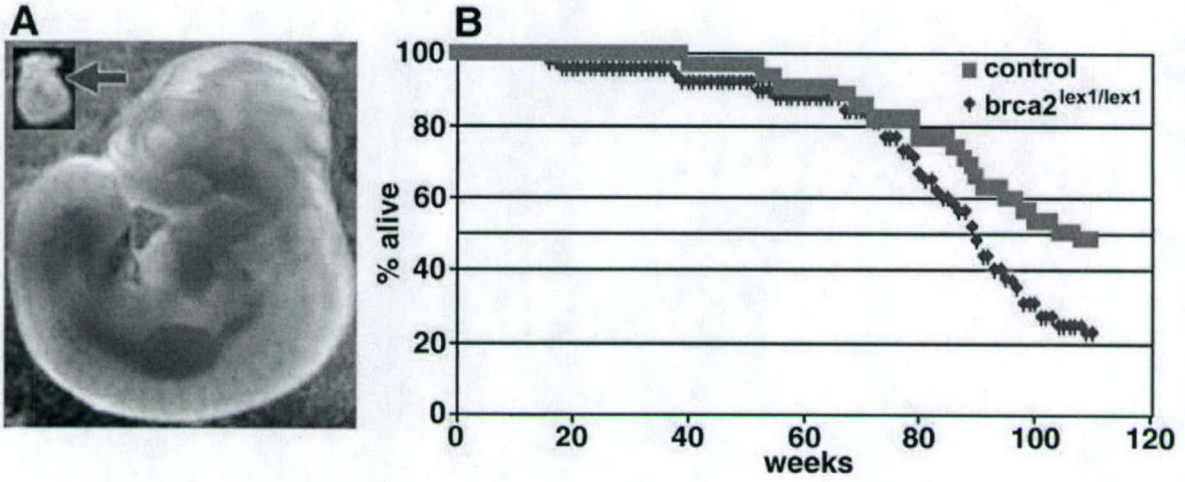

Figure 1. Effects of *Brca2*^{lex1} and *Brca2*^{lex2} alleles on viability. A. Day 6.5 BRCA2$^{lex2/lex2}$ embryo (inset; blue arrow) compared with an *Brca2*$^{+/lex2}$ *embryo. B. Survival curve of brca2*$^{lex1/lex1}$ (blue diamonds) and control (red squares) mice. The percentages of mice remaining alive are shown.

ing all of the BRC motifs) and still associates with RAD51 in the nucleus.

The progeny from *Brca2*$^{+/lex1}$ heterozygous breeding pairs were observed. There was an essentially normal Mendelian pattern of inheritance: of 195 progeny from heterozygous matings, 43 (22%) were *brca2*$^{lex1/lex1}$, 55 (28%) were *Brca2*$^{+/+}$, and 97 (50%) were *Brca2*$^{+/lex1}$. The *brca2*$^{lex1/lex1}$ mice did not exhibit any obvious phenotype compared to that of their control littermates (*Brca2*$^{+/+}$, *Brca2*$^{+/lex1}$). These mice were fertile, with no reduction in litter size. Because mice that are deleted for some of the BRC motifs are infertile (Connor et al., 1997; Friedman et al., 1998), this implies that the complete complement of BRC motifs in the BRCA2^{lex1} protein is necessary and sufficient for meiotic recombination. Similar results were observed from breeding of *Brca2*$^{+/lex1}$ heterozygotes with *Brca2*$^{+/lex2}$ heterozygotes. There was no reduction in the number of *brca2*$^{lex1/lex2}$ progeny observed, 21 out of a total of 62 (34%). These *brca2*$^{lex1/lex2}$ mice did not exhibit any obvious phenotype and were fertile. Thus, haploinsufficiency is not readily apparent in mice with one *brca2*^{lex1} allele and one *brca2*^{lex2} (null) allele.

Life Span and Pathology of *brca2*$^{lex1/lex1}$ and Control Mice

We compared the life span of 52 *brca2*$^{lex1/lex1}$ mice to 35 control mice (Fig. 1B). Included in the control cohort are *Brca2*$^{+/+}$, *Brca2*$^{+/lex1}$, and *Brca2*$^{+/lex2}$ mice. Heterozygous mice have shown no phenotypic abnormality in previous studies (data not shown). Mortality came sooner for the *brca2*$^{lex1/lex1}$ mice compared to control mice. The onset of mortality was about the same for *brca2*$^{lex1/lex1}$ and control mice, 50–60 weeks. However, the life-span curves began to diverge at about 70 weeks and were significantly different by 93 weeks (χ^2 test; $P < 0.05$). This divergence progressed and remained significant over each weekly interval until the study was terminated, when the youngest mice were 108 weeks old (P values ranging from <0.025 to 0.01). About 50% of the *brca2*$^{lex1/lex1}$ mice died by 89–90 weeks, whereas 50% of the control mice died by 104–108 weeks. Moribund mice (showing weight loss and marked decrease in mobility and responsiveness) and recently dead mice (found dead overnight or within several hours) were examined by necropsy. Additionally, all remaining mice were euthanized and examined by necropsy at the point where the survival curve reached 49% for the control population (108 weeks). All grossly abnormal tissues and potential tumors were examined by histopathology, as summarized in Table 1.

Both *brca2*$^{lex1/lex1}$ and control mice exhibited a wide range of cancers. However, onsets of cancer and sepsis (the latter indicated by reactive immune responses) were somewhat earlier in the *brca2*$^{lex1/lex1}$ mice. Reactive immune responses have previously been observed in mice deleted for the nonhomologous end-joining gene *Ku86*, and in wild-type mice as they reach the end of their life span (Vogel et al., 1999), and are therefore a common part of aging and not unique to the *brca2*$^{lex1/lex1}$

TABLE 1. Histopathology in *brca2*$^{lex1/lex1}$ and Control Mice

Mouse	Brca2 genotype	Age at death (weeks)	Histopathology
284H	lex1/lex1	67	Mesenteric abscess
246H	lex1/lex1	77	Colonic adenocarcinoma
107H	lex1/lex1	79	Hepatocellular carcinoma
109H	lex1/lex1	82	Fatty liver with focal inflammation
103H	lex1/lex1	86	Granulomatous inflammation in spleen
264H	lex1/lex1	87	Malignant lymphoma
142H	lex1/lex1	90	Reactive lymphoid hyperplasia in spleen
094H	lex1/lex1	96	Granulomatous inflammation in lymph nodes and skin
281H	lex1/lex1	98	Massive lymphoid hyperplasia in lymph nodes, possibly lymphoma
267H	lex1/lex1	101	Reactive lymphoid hyperplasia in lymph nodes, spleen, liver, kidney
050H	lex1/lex1	104	Hepatocellular carcinoma
256H	lex1/lex1	114	Osteosarcoma
022N	+/lex2	104	Hepatocellular carcinoma
028H	+/+	108	Leiomyosarcoma
051H	+/lex1	116	Hemangiosarcoma
053H	+/lex1	116	Hepatocellular carcinoma
058H	+/lex1	119	Malignant lymphoma
049H	+/lex1	129	Hepatocellular carcinoma

mice. From weeks 67 to 104, 11 *brca2*$^{lex1/lex1}$ mice (~20% of the population) developed life-threatening pathology (either cancer or sepsis), whereas the first control mouse to exhibit life-threatening disease did so at week 104. The cumulative incidence of cancers and reactive immune responses was significantly higher in the *brca2*$^{lex1/lex1}$ cohort compared to the control cohort by week 87 and continued to diverge through 108 weeks (χ^2 test; P values from <0.05 to 0.01). Although these differences seen through 108 weeks are statistically significant, they must be interpreted with caution, given the small numbers of animals involved. Nonetheless, the shortened life span of the *brca2*$^{lex1/lex1}$ cohort appears to be attributed, at least in part, to early onsets of cancer and sepsis.

Immortalized *brca2*$^{lex1/lex2}$ MEF Cells Show Reduced Viability and Hypersensitivity to DNA Damage

The shortened life span of *brca2*$^{lex1/lex1}$ mice and the early onsets of cancer/sepsis may be related to the inefficient recombinational repair and genomic instability previously seen in *brca2*$^{lex1/lex2}$ ES and MEF cells (Morimatsu et al., 1998; Moynahan et al., 2001b). To assess the cellular phenotype conferred by the *brca2*^{lex1} allele in greater depth, we performed further studies on a clone of immortalized *brca2*$^{lex1/lex2}$ MEFs and a clone of isogenic control MEFs (Morimatsu et al., 1998). In all experiments described below, the two clones were compared at approximately the same passage number (between 20 and 40). The immortalized *brca2*$^{lex1/lex2}$ MEFs could be propagated readily, but their growth was slower than that of isogenic wild-type immortalized cells. To determine whether cellular viability is impaired in the immortalized *brca2*$^{lex1/lex2}$ MEFs, we measured their cloning efficiency by colony-formation assays. In 11 paired determinations at various passage numbers, the mean cloning efficiency for wild-type cells was 24.9% (SD = 10.1%), but for the immortalized *brca2*$^{lex1/lex2}$ cells, only 16.0% (SD = 4.8%), that is, reduced by about one third. Although replicative senescence has been overcome in the immortalized *brca2*$^{lex1/lex2}$ MEFs, it appears that the original growth defect has not been entirely suppressed, given that their cloning efficiency is still significantly reduced relative to that of the wild-type MEFs (paired t-test; $P < 0.004$).

We previously showed that multiple clones of *brca2*$^{lex1/lex2}$ ES cells are more sensitive than wild-type ES cells to γ radiation, suggesting a defect in repair of DNA double-strand breaks (Morimatsu et al., 1998). To confirm that the immortalized *brca2*$^{lex1/lex2}$ MEF clone retains this phenotype, we assessed their sensitivity compared to that of immortalized isogenic wild-type MEFs. Sensitivity was measured by survival and proliferation (colony formation) after acute exposure to γ radiation at doses ranging from 2 to 10 Gy (Fig. 2A). At each dose tested, the viability of *brca2*$^{lex1/lex2}$ MEFs was significantly less than that for wild-type MEFs (P values ranging from 0.01 to 0.05). At 2 Gy, survival

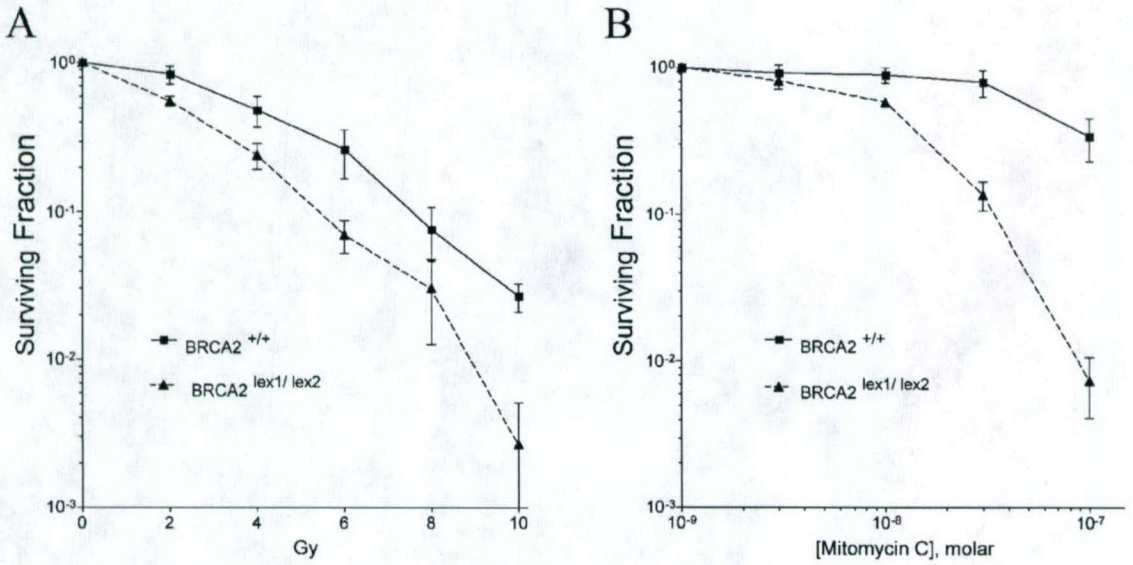

Figure 2. Genotoxic analysis of MEF cells. Sensitivity of *brca2*$^{lex1/lex2}$ and wild-type (*brca2*$^{+/+}$) immortalized MEF cells to γ radiation (**A**) or to mitomycin C (**B**) was measured by colony formation assays. Each data point represents the mean of three separate experiments. Each experiment was executed in triplicate. Error bars represent standard error of the mean. Note that the surviving fraction of cells in both panels is expressed on a logarithmic scale.

of *brca2*$^{lex1/lex2}$ MEFs was reduced by 1.5-fold relative to that of the wild-type. The difference became greater with increasing doses, so that at 10 Gy, survival of *brca2*$^{lex1/lex2}$ MEFs was reduced by 10-fold relative to that of the wild type. By interpolation, the LD_{50} for γ radiation (the dose at which 50% of cells are killed) is approximately 4.0 Gy for wild-type MEFs, but about 2.3 Gy for *brca2*$^{lex1/lex2}$ MEFs (i.e., reduced by roughly 40%). Thus, immortalized *brca2*$^{lex1/lex2}$ MEFs, like *brca2*$^{lex1/lex2}$ ES cells, show greater sensitivity to γ radiation.

Marked hypersensitivity to drugs that cause DNA interstrand crosslinks has been observed in mammalian cell lines defective in recombinational repair: the hamster *XRCC2*-mutant line irs1, the hamster *XRCC3*-mutant line irs1SF (Tebbs et al., 1995; Liu et al., 1998; Cui et al., 1999), and also in mouse cells with a more severe carboxy-terminal truncation in BRCA2 that removes some of the BRC motifs (Yu et al., 2000). We compared immortalized *brca2*$^{lex1/lex2}$ MEFs to immortalized wild-type MEFs for sensitivity to the DNA crosslinking drug mitomycin C. Sensitivity was measured by colony formation after plating in the presence of mitomycin C over a range of initial concentrations (Fig. 2B). The mean survival after plating in mitomycin C at 3×10^{-9} M (or less) was not significantly lower for *brca2*$^{lex1/lex2}$ MEFs than for wild-type MEFs. However, at progressively higher concentrations of mitomycin C (1×10^{-8} M, 3×10^{-8} M, and 1×10^{-7} M), the survival of *brca2*$^{lex1/lex2}$ MEFs fell sharply, and was significantly lower than that for the wild-type MEFs ($P < 0.02$, $P < 0.005$, and $P < 0.01$, respectively). At 3×10^{-8} M mitomycin C, the survival of *brca2*$^{lex1/lex2}$ MEFs was 5.6-fold lower, and at 1×10^{-7} M, 48-fold lower than for the wild-type MEFs. The LD_{50} for mitomycin C was approximately 5.7×10^{-8} M in wild-type MEFs, but about 1.1×10^{-8} M for *brca2*$^{lex1/lex2}$ MEFs (i.e., reduced by roughly 80%). Thus, immortalized *brca2*$^{lex1/lex2}$ MEFs are hypersensitive to mitomycin C.

Immortalized and Primary *brca2*$^{lex1/lex2}$ MEFs Exhibit Gross Chromosomal Instability

Elevated frequencies of chromosome breakage and rearrangement, either spontaneous or in response to DNA damage, have been seen in other mammalian cell lines known or thought to be defective for HRR, including hamster cell lines mutated for *XRCC2* or *XRCC3* (Tebbs et al., 1995; Liu et al., 1998; Cui et al., 1999), and in mouse cells with carboxy-terminal truncations that delete some of the BRC motifs of BRCA2 (Tutt et al., 1999; Yu et al., 2000). To assess the effects of deleting *Brca2* exon 27 on chromosome stability, we karyotyped immortalized *brca2*$^{lex1/lex2}$ and wild-type MEFs. Single clones of wild-type and *brca2*$^{lex1/lex2}$ MEFs

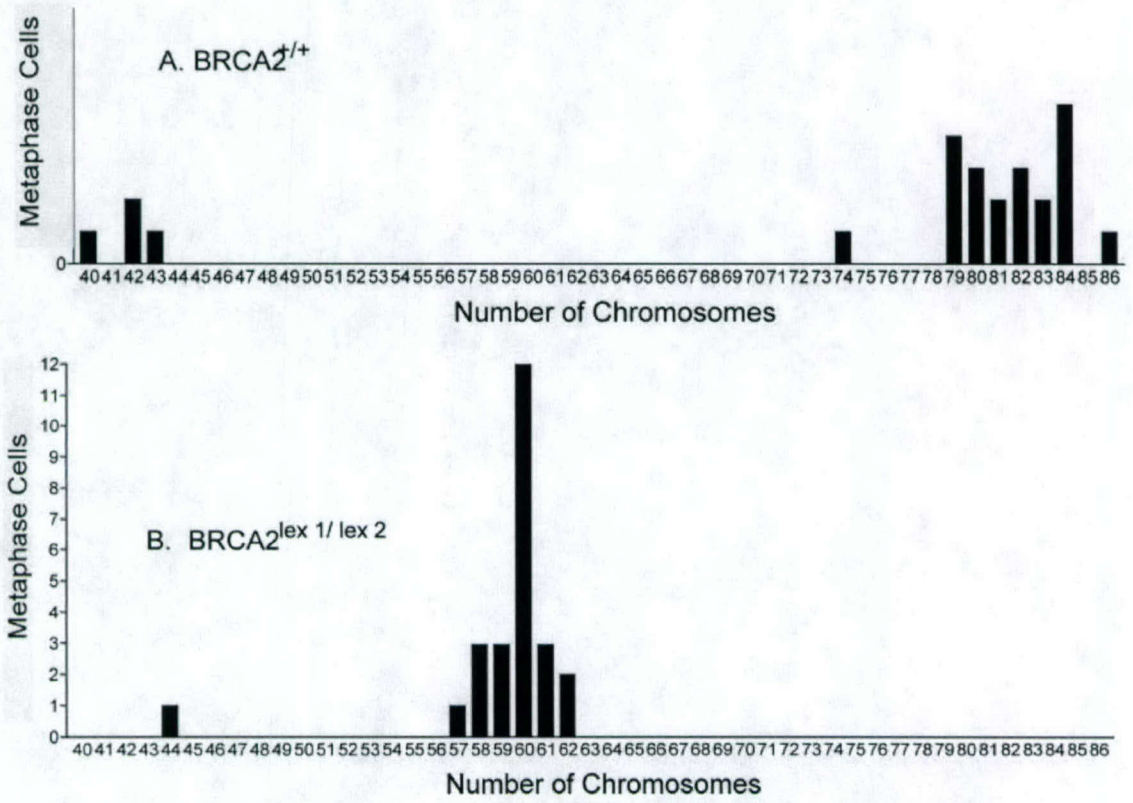

Figure 3. Chromosome number distributions in wild-type (**A**) and *brca2*$^{lex1/lex2}$ (**B**) immortalized MEF cells.

were harvested for metaphase cells at approximately equal periods of time in culture after their emergence as immortalized cell colonies (about 2 months; 20 passages). The wild-type and *brca2*$^{lex1/lex2}$ MEF lines differed markedly in chromosome numbers. The wild-type line showed a bimodal distribution of chromosome numbers, with most of the population approximating a tetraploid complement of 80 chromosomes, and a smaller part of the population near the diploid number of 40 chromosomes (Fig. 3A). Tetraploidization is commonly seen in spontaneously immortalized mouse cells within a few passages after their emergence from a primary cell population undergoing senescent crisis (Todaro and Green, 1963). The *brca2*$^{lex1/lex2}$ line, in contrast, was sub-tetraploid, with a modal chromosome number of 60 and a smaller subpopulation of near-diploid cells (Fig. 3B). One possible explanation for the lower numbers in the *brca2*$^{lex1/lex2}$ cell line is that they resulted from tetraploidization followed by a process of chromosome loss.

This interpretation is supported by additional evidence of chromosomal instability in *brca2*$^{lex1/lex2}$ MEFs. In comparison to wild-type MEFs, immortalized *brca2*$^{lex1/lex2}$ MEFs showed a significantly increased frequency of spontaneous chromatid-type aberrations (breaks, deletions, and exchanges; $P < 0.001$) and chromosome-type aberrations (mainly interstitial or terminal deletions; $P < 0.001$), as summarized in Table 2. Overall, spontaneous chromosomal abnormalities (chromatid and chromosome type) were about fourfold more frequent in *brca2*$^{lex1/lex2}$ MEFs. The immortalized *brca2*$^{lex1/lex2}$ MEFs also had a sharply increased occurrence of abnormally small chromosomes, or "minichromosomes," relative to that of the wild type. The *brca2*$^{lex1/lex2}$ MEFs averaged 4.7 minichromosomes per metaphase cell, with every metaphase cell examined displaying at least two. Wild-type MEFs averaged only 0.16 minichromosomes per metaphase cell. The metaphase chromosomes of immortalized MEFs were further examined by fluorescence in situ hybridization (FISH) by use of probes for telomeric repeats and for the mouse major satellite sequence, which lies in close proximity to the centromeres of mouse chromosomes. Representative *brca2*$^{lex1/lex2}$

TABLE 2. Spontaneous and Damage-Induced Chromosomal Aberrations in Immortalized Wild-Type and *brca2*$^{lex1/lex2}$ MEF Cells

MEF cells	Metaphases analyzed	Percentage aberrant metaphases[a]	Mean aberrations per metaphase[a]	Chromatid aberrations[b]				Chromosome aberrations[c]			
				Gaps	Breaks	Other	Total[a]	Int.del.	Ter.del.	Other	Total
Wild-type											
No treatment	30	50	0.800	5	9	2	11	9	3	1	13
γ-Irradiation	30	100	4.33	10	25	29	54	65	5	6	76
Mitomycin C	30	60	0.844	0	8	6	14	8	5	0	13
brca2$^{lex1/lex2}$											
No treatment	30	97	3.27	7	24	17	41	47	10	0	57
γ-Irradiation	30	100	5.07	17	35	24	59	75	0	18	93
Mitomycin C	30	93	4.60	13	66	30	96	27	14	1	42

[a]Calculated without chromatid gaps. [b]"Gaps" defined as discontinuities smaller than the width of the chromatid; "other" consisting of isochromatid deletions and exchanges. [c]Interstitial deletions; terminal deletions (including breaks); and other (including dicentric and ring chromosomes).

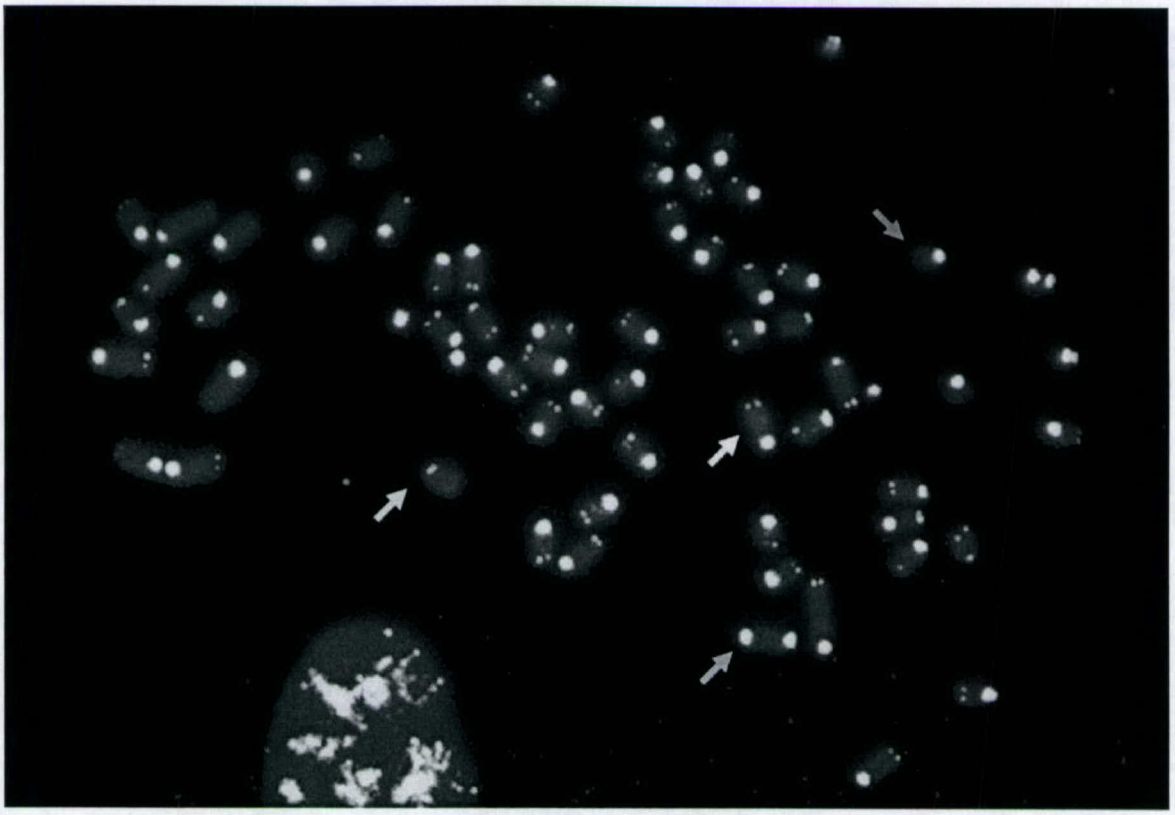

Figure 4. Fluorescence in situ hybridization with probes for centromeres and telomeres. Metaphase chromosomes prepared from immortalized MEF cells were hybridized with fluorescent probes for centromeric and telomeric sequences. The centromeric probe appears white in this composite image; the telomeric probe is green. Chromosomes were counterstained with DAPI (blue). Because normal mouse chromosomes are acrocentric, only the telomeres of the long arms can be seen here. Telomeres of the short arms are obscured by the brighter centromeric probe. An example of a normal chromosome is indicated by the **white arrow**. Most of the minichomosomes seen in the *Brca2*-mutant metaphase cells lacked detectable telomeres; at least five of these occur in this metaphase (an example is indicated by the **red arrow**). Acentric and dicentric chromosomes or minichromosomes were also seen frequently in *Brca2*-mutant metaphase cells. Four acentrics (e.g., **yellow arrow**) and two dicentrics (e.g., **green arrow**) can be seen in this metaphase.

metaphase chromosomes are shown in Figure 4. Most of the minichromosomes seen in immortalized *brca2*$^{lex1/lex2}$ MEFs lacked detectable telomeres, and of the minority that retained telomeres, most lacked a detectable centromeric region. This implies that the minichromosomes are actually chromosome frag-

ments. In addition to acentrics, dicentric minichromosomes were also present in many *brca2*$^{lex1/lex2}$ MEF metaphase cells, but were seen very rarely in wild-type MEFs. Chromosome fragments that lack centromeres are subject to loss by segregation errors, whereas chromosome fragments that lack telomeres can be expected to undergo progressive shortening and further cycles of fusion and breakage events. The frequent occurrence of dicentric minichromosomes in immortalized *brca2*$^{lex1/lex2}$ MEFs may reflect the latter process.

To assess the relative effects of DNA damage on chromosomal integrity in wild-type and *brca2*$^{lex1/lex2}$ immortalized MEFs, we prepared metaphase cells 24 hr after exposure to 6 Gy of γ radiation. After γ irradiation, there were significantly increased numbers of chromatid- and chromosome-type abnormalities in *brca2*$^{lex1/lex2}$ MEFs relative to nonirradiated controls, but this was also true for wild-type MEFs (Table 2). The numbers of chromatid and chromosome aberrations present in *brca2*$^{lex1/lex2}$ MEFs after irradiation were only slightly higher than those in wild-type MEFs, suggesting that *brca2*$^{lex1/lex2}$ cells are nearly as proficient as wild-type cells in repairing the chromosomal damage induced by ionizing radiation.

Chromosomal integrity was also examined 24 hr after application of mitomycin C at 5×10^{-8} M. In wild-type MEFs, this exposure produced no significant increase in chromatid- or chromosome-type abnormalities (Table 2), suggesting that cells expressing normal BRCA2 were able to repair most of the interstrand crosslinks created. In *brca2*$^{lex1/lex2}$ MEFs, however, the frequency of chromatid-type aberrations increased more than twofold after mitomycin C exposure (Table 2, $P < 0.001$). Chromatid-type aberrations (metaphase chromosomes in which only one sister chromatid exhibits a defect) are presumed to represent damage that occurred after (or during) the immediately preceding round of replication. The result implies that repair of interstrand crosslinks occurring in replicated (or replicating) DNA is impaired in *brca2*$^{lex1/lex2}$ cells, but does not necessarily prevent them from entering metaphase. Mitomycin C exposure produced no increase in chromosome-type aberrations in either wild-type or *brca2*$^{lex1/lex2}$ MEFs. Chromosome-type aberrations (in which both sister chromatids exhibit the same defect) are presumed to originate with damage occurring before replication, or in a previous cell cycle. The lack of excess chromosome-type aberrations after mitomycin C exposure may indicate that cells suffering interstrand crosslinks during interphase (when no sister chromatid is available as a template for HRR) repair them with low efficiency and usually fail to progress through S phase to metaphase.

We sought to determine whether the chromosomal instability seen in immortalized *brca2*$^{lex1/lex2}$ MEFs arose during the process of immortalization, or already existed in primary cells before senescent crisis. We therefore examined early-passage primary MEFs for numerical and structural abnormalities. Two populations of *brca2*$^{lex1/lex2}$ MEFs and three populations of wild-type MEFs (Morimatsu et al., 1998) that had been frozen at the first passage after dissociation from embryos (passage 1) were thawed and passaged, with metaphase cells prepared at each passage. All clones of passage 1 wild-type MEFs grew vigorously after thawing, and showed no appreciable slowing of growth over at least 10 passages afterward (data not shown). However, both *brca2*$^{lex1/lex2}$ MEF lines senesced prematurely in culture, as reported previously (Morimatsu et al., 1998), and could not be expanded beyond the third (line 281.1) or fourth passage (line 283.2). In contrast to their immortalized counterparts, primary *brca2*$^{lex1/lex2}$ MEFs did not differ significantly in chromosome numbers from those of the wild type. Average chromosome numbers were essentially diploid in both *brca2*$^{lex1/lex2}$ (mean = 39.97) and wild-type (mean = 40.00) primary MEFs and did not change significantly in either population over the few passages observed. Chromosome loss may be constrained in diploid cells, such that cells that lose multiple chromosomes suffer reduced viability and are quickly lost from the population. Chromosome numbers were, however, significantly more variable in primary *brca2*$^{lex1/lex2}$ MEFs (SD = 0.5237) than in the wild type (SD = 0.2912; F test for difference in variance; $F = 3.255$, $P < 0.001$). Distributions of chromosome numbers in primary *brca2*$^{lex1/lex2}$ and wild-type MEFs are graphed in Figure 5. The more frequent presence of cells with missing or extra chromosomes in primary *brca2*$^{lex1/lex2}$ MEFs suggests an impaired ability to segregate chromosomes faithfully. Scoring of early-passage primary MEFs for structural chromosomal aberrations is summarized in Table 3. Compared to wild-type primary MEFs, the *brca2*$^{lex1/lex2}$ primary MEFs had sharply elevated numbers of chromatid and chromosome aberrations, including gaps, breaks, deletions, and exchanges. The *brca2*$^{lex1/lex2}$ MEFs had higher numbers of chromosomal aberrations even at passage 2, and accumulated further aberrations more rapidly than did wild-type MEFs. By the third passage, approximately half the metaphase cells in

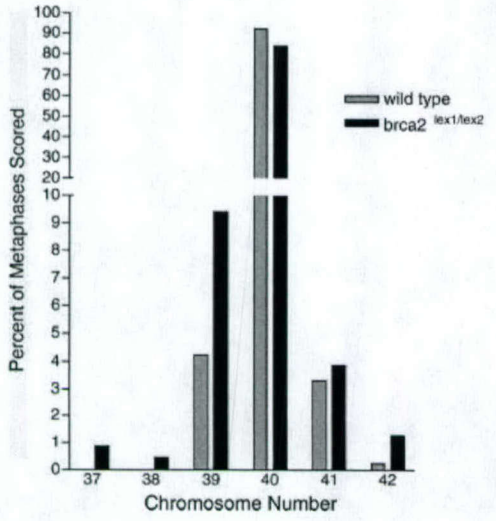

Figure 5. Chromosome number distributions in wild-type and *brca2*$^{lex1/lex2}$ primary MEF cells.

brca2$^{lex1/lex2}$ populations had one or more visible chromosome abnormalities. These results confirm that the chromosomal instability seen in immortalized *brca2*$^{lex1/lex2}$ MEFs results directly from the *brca2*$^{lex1/lex2}$ mutation, and not from additional mutations acquired during or after immortalization.

DISCUSSION

brca2$^{lex1/lex2}$ ES cells are defective for homologous recombinational repair of specifically induced chromosomal double-strand breaks (Moynahan et al., 2001b). The cellular and animal phenotypes described here can be interpreted in light of this repair defect. Both *brca2*$^{lex1/lex2}$ ES cells and immortalized MEFs are more sensitive than their wild-type counterparts to γ radiation, but this hypersensitivity is mild compared with that conferred by mutations known to affect the nonhomologous end-joining pathway for double-strand break repair (Ku80 or DNA-PKcs) (Caldecott and Jeggo, 1991; Collins, 1993). This is consistent with the idea that nonhomologous end-joining is the predominant pathway for repair of radiation-induced double-strand breaks in mammalian cells, whereas homologous recombination plays a comparatively minor role in repairing this form of damage. However, this observation may also reflect that homologous recombination is diminished, but not ablated, in *brca2*$^{lex1/lex2}$ cells (Moynahan et al., 2001b).

Immortalized *brca2*$^{lex1/lex2}$ MEFs are hypersensitive to the DNA crosslinking drug mitomycin C. Mitomycin C can form monoadducts with DNA, and intrastrand as well as interstrand crosslinks. Monoadducts and intrastrand crosslinks involve bases on only one strand of the DNA duplex and can be repaired by nucleotide excision repair in much the same way as other bulky adducts or UV-induced pyrimidine dimers (Friedberg et al., 1995). We previously found that *brca2*$^{lex1/lex2}$ ES cells had no hypersensitivity to UV irradiation (Morimatsu et al., 1998). Repair of interstrand crosslinks, however, necessarily involves cleavage and processing of both strands and hence the potential for loss of genetic information. In *Escherichia coli*, interstrand crosslink repair is accomplished without loss of information by a pathway that includes elements of both nucleotide excision repair and homologous recombination (Friedberg et al., 1995; Dronkert and Kanaar, 2001). The process is dependent on the bacterial RecA protein, the prototypical recombinational strand transferase to which the Rad51 family of eukaryotic recombination and repair proteins is related. There is substantial evidence that repair of interstrand crosslinks in mammalian cells also involves both nucleotide excision and recombinational repair functions (Friedberg et al., 1995; Thompson and Schild, 1999; Dronkert and Kanaar, 2001). The results obtained here suggest that BRCA2 participates in this process and that the RAD51-interacting domain encoded by exon 27 is important for full activity.

Brca2$^{lex1/lex2}$ MEFs are genomically unstable at the chromosomal level. This is likely to be a direct consequence of their defect in HRR. Evidence of gross chromosomal rearrangements, breakage, and/or chromosome loss has been observed in mutant cells and cell lines for several other genes with confirmed or strongly suspected involvement in HRR, including *RAD51* (Lim and Hasty, 1996; Sonoda et al., 1998) and its paralogs (Tebbs et al., 1995; Liu et al., 1998; Cui et al., 1999; Takata et al., 2000, 2001), *MRE11* (Yamaguchi-Iwai et al., 1999), *NBS1* (Petrini et al., 2001), and the other major breast cancer susceptibility gene, *BRCA1* (Shen et al., 1998; Xu et al., 1999; Moynahan et al., 2001). Thus, gross chromosomal instability has emerged as a characteristic phenotype of mutations that compromise HRR. How defective HRR results in chromosome aberrations is not known with certainty. They may arise from failure to initiate repair in some instances, resulting in persistent chromosome breaks, or by the default of unrepaired or abortively repaired damage into more error-prone nonhomologous mechanisms, producing rearrangements.

TABLE 3. Spontaneous Chromosomal Aberrations in Early-Passage Wild-Type and *brca2*$^{lex1/lex2}$ Primary MEF Cells

MEF cells	Passage	Metaphases analyzed	Percentage aberrant metaphases[a]	Mean aberrations per metaphase[a]	Chromatid aberrations[b]				Chromosome aberrations[c]			
					Gaps	Breaks	Other	Total[a]	Int.del.	Ter.del.	Other	Total
Wild-type												
129.5	P2	50	6	0.06	7	1	0	1	0	2	0	2
	P3	50	6	0.06	13	1	0	1	0	1	1	2
	P4	50	10	0.10	10	1	0	1	0	2	2	4
129.7	P2	50	4	0.06	4	3	0	3	0	0	0	0
	P3	50	8	0.12	1	1	1	2	2	2	0	4
	P4	50	14	0.24	3	5	0	5	2	5	0	7
129.9	P2	50	4	0.04	0	0	0	0	0	2	0	2
	P3	50	8	0.08	1	2	0	2	0	2	0	2
	P4	50	26	0.28	7	3	2	5	3	5	1	9
brca2$^{lex1/lex2}$												
283.2	P2	50	24	0.40	22	11	2	13	0	7	0	7
	P3	50	58	2.22	22	50	6	56	23	32	0	55
	P4	42	48	1.02	14	18	4	22	2	27	0	29
281.1	P2	50	52	0.76	5	23	0	23	5	10	0	15
	P3	40	62	1.60	23	35	5	40	5	19	0	24

[a]Calculated without chromatid gaps.

[b,c]"Gaps" defined as discontinuities smaller than the width of the chromatid; "other" consisting of isochromatid deletions and exchanges.

[c]Interstitial deletions; terminal deletions (including breaks); and other (including dicentric and ring chromosomes).

Mutation of genes involved in nonhomologous end-joining has been associated with fusion of chromosomes at the telomeres (Bailey et al., 1999; Hsu et al., 2000; Samper et al., 2000). However, FISH analysis of *brca2*$^{lex1/lex2}$ MEFs has shown no telomeric fusions (S. Bailey and E. H. Goodwin, unpublished data), and therefore does not support any role for BRCA2 in protection or "capping" of telomeres.

Impaired HRR may not be the only cause of chromosomal instability in BRCA2-deficient cells. In human cells, dominant-negative inhibition of BRCA2 has been associated with impairment of the G2/M cell cycle checkpoint (Chen et al., 1999). Such a defect might contribute to chromosomal instability by permitting cells with unrepaired chromosome breaks to enter mitosis. Mutation of BRCA2 in mouse cells has also been associated with abnormal centrosome function, that is, extra centrosomes or centrosome fragments that produce multipolar mitotic spindles and incorrectly segregated chromosomes (Tutt et al., 1999). Similarly, defective centrosome function has been seen in HRR-deficient XRCC2- and XRCC3-mutant hamster cells (Griffin et al., 2000). Checkpoint defects were not detected in *brca2*$^{lex1/lex2}$ MEFs (Morimatsu et al., 1998), but, because these cells have not been examined for abnormal centrosomes, it remains possible that centrosome defects contribute to the chromosomal instability that we observed.

The phenotype of *brca2*$^{lex1/lex2}$ ES cells and MEFs can be compared with those of mouse cells bearing other sublethal *Brca2* mutations. One mutation targeted within exon 11 truncates the *Brca2* open reading frame near the 3′ end of exon 11, preserving the first six of the eight BRC repeats and part of the seventh [*brca2*^{Tr2014} (Connor et al., 1997; Tutt et al., 1999)]. Primary MEFs homozygous for this mutation show a growth defect in culture, and overexpress both p21 and p53. These cells are also deficient in repair of ionizing radiation-induced double-strand breaks, as assessed by comet assay, and show elevated frequencies of chromosome breakage. A second mutation targeted within exon 11 retains only the first three BRC motifs (Patel et al., 1998; Yu et al., 2000). Primary MEFs homozygous for this mutation also suffer a proliferative defect, as well as increased sensitivity to ultraviolet radiation, methyl-methanesulfonate, or mitomycin C, and a high frequency of spontaneous chromosomal breaks and aberrant chromatid exchanges.

Together with the results reported here, these findings indicate that mutations in either of the regions of the BRCA2 protein shown to mediate interaction with RAD51 (the BRC repeats and exon 27) can produce cellular phenotypes of growth impairment, sensitivity to DNA damage, and gross chromosomal instability. It should be noted that, although the more severe BRCA2 truncations that omit some BRC repeats produce phenotypes consistent with defective HRR, their impact on HRR has not been directly assessed, apparently because of difficulty in deriving immortalized cell lines homozygous for these mutations. Recently, compound heterozygous ES cells with the*brca2*^{Tr2014} allelle and a targeted deletion of *Brca2* exon 27 have been developed (Tutt et al., 2001) and found to have a defect in HRR by gene conversion similar to that reported for *brca2*$^{lex1/lex2}$ ES cells (Moynahan et al., 2001b), together with gross chromosomal instability similar to that described here.

Although the cellular phenotypes of the *brca2*^{lex1} truncation and the more severe carboxy-terminal BRCA2 truncations that delete some of the BRC motifs are qualitatively similar, the mouse phenotypes are very different. Mice homozygous for either of the severe carboxy-terminal truncations exhibit partial preadulthood lethality, infertility, runted growth, and a high incidence of thymic lymphoma before 6 months of age (Connor et al., 1997; Friedman et al., 1998; Patel et al., 1998). However, mice that are homozygous for the *brca2*^{lex1} deletion appear relatively normal. Nearly all reach adulthood and are fertile, much like their control littermates, yet their life span is significantly shorter than that of control mice, possibly attributable in part to early onsets of cancer and sepsis. That the larger carboxy-terminal truncations result in severe phenotypes is perhaps not surprising, given that they eliminate more than a third (Connor et al., 1997) or more than half (Patel et al., 1998) of the BRCA2 protein. The large carboxy-terminal region lost in these mutations is known to contain interaction domains for at least three proteins other than RAD51: DSS1, BCCIPa, and filamin-1 (Marston et al., 1999; Liu et al., 2001; Yuan and Shen, 2001), and may contain other domains with important functions as well, so that the resulting phenotypes cannot be ascribed with certainty to the loss of RAD51-interacting regions alone. The *brca2*^{lex1} allele deletes less than 6% of the protein and leaves all eight of the BRC repeats, as well as the intervening region coded by exons 12–26, intact. Moreover, the truncated BRCA2 pro-

tein encoded by the *brca2*^{lex1} allele retains the capacity to bind RAD51, presumably through the BRC repeats (Moynahan et al., 2001b). Thus, although the more severe phenotypes seen in mice with longer carboxy-terminal truncations may be the result of more severely impaired HRR, it is also possible that they reflect loss of functions not related to RAD51.

Recently, another group reported the phenotype of mice with a deletion of exon 27, called *brca2*$^{\Delta 27}$ (McAllister et al., 2002). Homozygosity for the *brca2*$^{\Delta 27}$ allele resulted in a more severe phenotype than reported here for *brca2*$^{lex1/lex1}$ mice, including significant late embryonic or neonatal lethality (about 33%), altered mammary ductal morphology, and increased tumor incidence (61% of mice developed tumors, as compared to 20–26% of controls). There are several possible explanations for this marked difference in phenotypic severity. First, the genetic background may influence the phenotype. Although the *brca2*$^{\Delta 27/\Delta 27}$ mice and *brca2*$^{lex1/lex1}$ mice were created on a similar genetic background (129/C57Bl), the 129 mouse strain has been shown to diverge readily (Simpson et al., 1997). Second, the *brca2*$^{\Delta 27}$ allele may produce lower levels of protein, or a protein that differs from the *brca2*^{lex1} protein at the carboxy terminus. In construction of the *brca2*^{lex1} allele, a splice acceptor, stop codon, and polyadenylation signal were provided to replace exon 27. This allele has been shown to produce the predicted truncation transcript (Morimatsu et al., 1998) and truncated protein, at approximately wild-type levels (Moynahan et al., 2001b). The truncated BRCA2^{lex1} protein has a single added amino acid (Asp) after exon 26. The targeting scheme used to generate the *brca2*$^{\Delta 27}$ allele makes no provision for a new splice acceptor, and it is not clear exactly how the *brca2*$^{\Delta 27}$ transcript would acquire a stop codon and polyadenylation signal. The *brca2*$^{\Delta 27/\Delta 27}$ mice were not characterized for levels of transcript or the levels or subcellular location of protein. Although it seems certain that the *brca2*$^{\Delta 27}$ allele is expressed (because *brca2*$^{\Delta 27/\Delta 27}$ mice survive), recruitment of a new 3′ end may alter the stability and/or processing of the transcript such that the expression level is reduced. Acquisition of a new stop codon would add an unknown number of amino acids to the carboxy terminus with unknown effects on function.

The results obtained here confirm that the extreme carboxy-terminal region encoded by exon 27, although not essential to viability in mice, is important for the function of BRCA2 at the cellular level. Interaction between the extreme carboxy terminus of murine BRCA2 and RAD51 was originally reported by Sharan et al. (1997) and by Mizuta et al. (1997), both of whom detected the interaction of small carboxy-terminal fragments of BRCA2 (273 amino acids and <200 amino acids, respectively). Although a later study failed to detect interaction between RAD51 and the carboxy-terminal region of murine BRCA2 (Sarkisian et al., 2001), this may be related to the much larger mutant BRCA2 peptide used (omitting only exon 11), which might, for example, fold differently. More recent studies have confirmed that the RAD51/exon 27 domain interaction is conserved by the human proteins (X. Guo and Z. Shen, personal communication). It remains a formal possibility that the phenotype associated with deletion of exon 27 results from something other than altered interactions with RAD51. The human BRCA2 protein is dependent on nuclear localization signals encoded within exon 27 for transport into the nucleus (Spain et al., 1999). However, this is evidently not the case for mouse BRCA2 because a truncation retaining only the most amino-terminal fourth of the protein efficiently localizes to the nucleus when expressed in human cells (Sarkisian et al., 2001). Thus, in the mouse, interaction with RAD51 is the only function demonstrated for the region encoded by exon 27. This raises the interesting question of why the BRCA2 protein should require two distinct kinds of RAD51-interaction domains (the BRC repeats and the exon 27 domain) to be fully functional.

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A genotoxic screen: rapid analysis of cellular dose–response to a wide range of agents that either damage DNA or alter genome maintenance pathways☆

Teresa Marple, Han Li, Paul Hasty*

The Department of Molecular Medicine and Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, 15355 Lambda Drive San Antonio, TX 78245-3207, USA

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Abstract

SNP analysis has come to the forefront of genomics since the mouse and human genomes have been sequenced. High throughput functional screens are necessary to evaluate these sequence databases. Described here is a genotoxic screen: a rapid method that determines the cellular dose–response to a wide range of agents that either damage DNA or alter basic cellular pathways important for maintaining genomic integrity. Importantly, a single person utilizing standard tissue culture equipment may perform these assays composed of 20 agents that attack genomic integrity or maintenance at many different levels. Thus, a small lab may perform this screen to determine the integrity of a wide range of DNA repair, chromatin metabolism, and response pathways without the limitations of investigator bias. A genotoxic screen will be useful when analyzing cells with either known genetic alterations (generated directly by the investigator or derived from individuals with known mutations) or unknown genetic alterations (cells with spontaneous mutations such as cancer-derived cells). Screening many genotoxins at one time will aid in determining the biological importance of these altered genes. Here we show the dose–response curves of mouse embryonic stem (ES) cells and HeLa cells exposed to 20 genotoxic agents. ES cells were chosen since they are amenable to genetic alteration by the investigator. HeLa cells were chosen since they were derived from cancer and are commonly used. Comparing the dose–response curves of these two cell lines show their relative sensitivity to these agents and helps define their genotoxic profile. As a part of phenomics, a large genotoxic profile database for cancer-derived cells, when integrated with other databases such as expression profiles and comparative genomic hybridization, may aid in maximizing the effectiveness of developing anti-cancer protocols.

Keywords: Anti-cancer therapeutics; Chemotherapy; Dose–response; Drug resistance; Hypersensitivity

1. Introduction

A dose–response curve displays a cell's ability to thrive (live and proliferate) in the presence of certain damaging agents. With increasing dose, fewer cells thrive. Cells may be exposed to a variety of agents that damage the cell in very specific ways. For this pa-

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* Corresponding author. Tel.: +1 210 567 7278; fax: +1 210 567 7247.
E-mail address: hastye@uthscsa.edu (P. Hasty).

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per, genotoxic agents are tested; these agents damage DNA, alter chromatin metabolism or impact the responses to these challenges. Even though many genotoxic agents damage more than DNA, dose–response curves have accurately identified the biological importance of altered DNA repair genes [1]. Thus, damage to macromolecules other than DNA is likely about the same for the control cells as for the DNA repair deficient cells. As a result, the dose–response curve reveals cellular phenotype that suggests function for an altered gene. Therefore, by testing a wide range of agents, gene product function may be assessed without the limitations of investigator bias.

This nonbiased approach may be important since there are many chromatin metabolism pathways that maintain genomic instability and these pathways are not completely understood. These pathways include repairing lesions to single strands: base excision repair (BER) [2], nucleotide excision repair (NER) [3] and transcription-coupled repair (TCR) [4]. Also included are pathways that repair lesions to double strands: homologous recombination repair (HRR) [5], nonhomologous endjoining (NHEJ) [6] and interstrand cross-link repair [7]. Genome stability is also ensured by a set of replication error correction systems that include mismatch repair (MMR) [8], helicases [9], topoisomerases [10], and replicative bypass DNA polymerases [11]. Additionally, telomerase is a protein-RNA complex that caps chromosomal ends to form a telomere that protects the end against degradation, end-to-end fusion and recombination [12]. Impairment of these pathways may be detected by increased sensitivity to agents that cause the type of damage repaired by the defective pathway.

There are a host of pathways that indirectly maintain genome stability by responding to DNA damage or errors in chromosomal metabolism [13,14]. These pathways may either induce programmed cell death (apoptosis) or halt cell cycle progression (cell cycle checkpoints) [15]. Impairment of these pathways may alter resistance to some genotoxic agents; for example deletion of the tumor suppressor gene p53 increases resistance to a variety of genotoxic agents [16,17].

A genotoxic screen was performed on mouse embryonic stem (ES) cells and HeLa cells. This screen is composed of 20 agents that either mutate DNA or alter basic pathways important for genomic integrity and has been optimized for speed and cost. Determining the dose–response to all of these agents will help elucidate the function for altered genes without the limitations of investigator bias. The genotoxic profiles for ES cells and HeLa cells are compared.

2. Materials and methods

2.1. Cell culture

Mouse ES cells were maintained in M15: high glucose DMEM supplemented with 15% fetal bovine serum, 100 μM β-mercaptoethanol, 1 mM glutathione, 3 mg/ml penicillin, 5 mg/ml streptomycin, 1000 U/ml ESGRO (LIF) and grown on plastic coated with gelatin (0.1%) for at least 1 h. Lex1 ES cells (129SvEvBrd cells, Hprt positive, from Stratagene) were used for all assays. In addition, AB2.2 ES cells (129SvEvBrd cells, Hprt negative) were used for the 6-thioguanine assay. HeLa cells (from ATCC) were maintained in M10: high glucose DMEM supplemented with 10% fetal bovine serum, 1 mM glutathione, 3 mg/ml penicillin and 5 mg/ml streptomycin. Cells were grown in 5% CO_2 in a 37 °C incubator.

2.2. Genotoxic screen

ES cells and HeLa cells were expanded onto a 10 cm plate for the genotoxic screen. After the total number of cells needed for the experiment was taken, media was added to a final concentration of 600 cells/ml for ES cells and 1000 cells/ml for HeLa cells. One milliliter of cells was added per well for the eight inner wells of a 24-well plate. Two milliliters of PBS was added to the outer 16 wells to reduce evaporation because evaporation will artificially reduce cell number which is more of a problem for the outer wells than the inner wells (evaporation is a problem after replacing the 1 ml of medium with 250 μl of genotoxin). This is considered day 0. Those chemical agents that must be dissolved in solvent were added the following day, 24–30 h following seeding (Table 1). This is considered day 1. Genotoxins were mixed with the appropriate solvent (Table 1) at the highest concentration used and serial dilutions were made from this solution. For each well, the 1 ml of media was replaced with 250 μl

Table 1
Solvent and storage conditions for the genotoxic agents

Genotoxin	Classification	Solvent	Stock[a]	Solvent[b] (%)	storage (°C)
γ-Radiation	Natural	None	NA	NA	NA
UVC		None	NA	NA	NA
H_2O_2	ROS	Water	7 M	2	4
Streptonigrin		DMSO	100 mM	0.9	−20
Paraquat		Water	1 M	2	−20
NAC		Water	1 M	2	−20
Ebselen		DMSO	30 mM	0.9	−20
MMS	Alkylating	Water	30 mM	2	−20
ENU		DMSO	6 M	0.9	−20
Cisplatin	Cross-linking	DMSO	400 μM	0.9	−20
MMC		PBS	10 mM	2.1	4
Camptothecin	Topo inhibtors	DMSO	50 mM	0.9	−20
ICRF-193		DMSO	1 mM	0.9	−20
Etoposide		DMSO	300 mM	0.9	−20
HU	CCCM	Water	1 M	2	−20
Aphidicolin		DMSO	30 mM	0.9	−20
6-TG		PBS pH 9	10 mM	2.9	−20
L-Mimosine		PBS pH 9	50 mM	2.9	−20
Colcemid		PBS	10 mM	2.1	−20
TSA		DMSO	600 μM	0.9	−20

NAC, *N*-acetyl-L-cysteine; HU, Hydroxyurea; MMS, Methyl methane sulfonate; MMC, Mitomycin C; 6-TG, 6-Thioguanine; ENU, *N*-ethyl-*N*-nitrosourea; TSA, Trichostatin A; UVC, ultraviolet light C; DMSO, dimethyl sulfoxide; CCM, chromosomal/cell cycle metabolism; NA, not applicable.

[a] Concentration of stock solution.

[b] Maximum final concentration of solvent used for the assay.

media with genotoxin. All genotoxins were obtained from Sigma with the exception of ICRF-193 (ICN Biochemicals).

The conditions are different for the two agents that do not require a solvent, γ-radiation and UVC light. For ionizing radiation, ES cells (2400 cells/ml) and HeLa cells (4000 cells/ml) were exposed to ^{137}Cs (Mark1 gamma radiation source from Shepard and Associates) at a rate of 0.125 Gy/s in 3 ml of medium in a 15 ml tube. These cells were plated at 250 μl/well for the inner eight wells of a 24-well plate (2 ml PBS was added into the outer 16 wells to reduce evaporation). The zero dose control cells were placed in the radiation chamber for the same amount of time as required for the highest dose, but were not irradiated. Thus, cells are exposed to ionizing radiation on day 0 (cells are exposed on day 1 for all other agents). For UVC light, a colony-forming assay was performed using a 6-well plate. A colony-forming assay was used since the walls of a well on a 24-well plate are high compared to the surface area and these walls block most of the UVC light. Therefore, ES cells were seeded 1200 cells per well and HeLa cells were seeded 2000 cells per well of a 6-well plate in 2 mls medium (day 0). Twenty-four to thirty hours after seeding, the medium was removed and plates were exposed to UVC light at a rate of 1 J/m^2/s using a duel wavelength UV transilluminator (Alpha Innotech Corp.). Medium was removed from control wells for the amount of time corresponding to the highest dosage of UVC (14 s). After exposure the wells were replenished with 2 ml fresh medium.

Cells, not colonies, were counted with a hemacytometer in the presence of trypan blue after exposed to all genotoxins, except UVC. ES cells were harvested on day 6 and HeLa cells were harvested on day 7. For cells exposed to UVC, ES cell and HeLa cell colonies were counted on day 8. The survival fraction (SF)

is determined by the number of viable exposed-cells (colonies for UVC) divided by the number of viable nonexposed cells (colonies for UVC).

2.3. Time course after exposure to camptothecin

HeLa cells and ES cells were analyzed by a time course with fixed concentrations of camptothecin (supplement 1.1). Cells were counted with a hemacytometer and then analyzed for proliferation (supplement 1.1.1), apoptosis (supplement 1.1.2) and DNA content (supplement 1.1.3) using a BD FacsCaliber flow cytometer. Both a 24-well format and a 6-well format were used.

3. Results

Our goal is to present a rapid, cost-effective protocol to measure cellular resistance to 20 genotoxins that will test a variety of pathways that repair DNA, influence chromatin metabolism or responses to alterations in DNA (Table 1). In order to minimize cost (genotoxins can be expensive), we perform 19 of the 20 assays in the wells of 24-well plates (15 mm) with a total of 250 μl media per well. In addition, a single person may perform these experiments using common tissue culture equipment. Thus, this screen is designed for any small to average-size lab that has access to a standard tissue culture room.

3.1. Experimental design

These dose–response assays measure viable cells after exposure to different genotoxins. There are 18 genotoxins that are chemicals dissolved in solvent and two genotoxins that are not chemicals in solution (γ-radiation and UVC light) as described in Table 1. With the exception of the UVC assay (see Section 2), the total cell number is counted, not the number of colonies. Therefore, this is a dynamic assay that must be customized according to proliferation rates of the cells being tested. It is important to seed cells at a density that permits consistent proliferation throughout the test. A seeding density and harvest day was chosen based on the slop of the growth curve; the slope must be linear on a logarithmic scale (see supplement 1.2 and supplement Fig. 1). Seeding densities of 600 ES cells/15 mm well and of 1000 HeLa cells/15 mm well were chosen (the wells of a 24-well plate are 15 mm in diameter). Harvest day 6 and 7 was chosen for ES cells and HeLa cells, respectively (cells are seeded on day 0). These seeding densities and harvest days analyze cells in a strong growth phase that is not compromised by overcrowding.

3.2. The genotoxic screen for ES and HeLa cells

A comprehensive test of the varied DNA repair and chromatin metabolism pathways in a cell requires multiple genotoxic agents that cause a wide range of lesions [1,18]. We have determined genotoxin dosages that reduce cell number by 0–99.9% for ES and HeLa cells exposed to 20 genotoxins with special attention given to doses that reduce cell number by 1–90%. These experiments have been repeated, on different days, for both ES and HeLa cells exposed to all genotoxins (error bars are shown; however, frequently the error bars are smaller than the symbol). The following genotoxins are placed into six groups based on mode of action; however, placement is somewhat arbitrary. These groups are (1) naturally occurring exogenous agents, (2) ROS-altering agents, (3) alkylating agents, (4) cross-linking agents, (5) topoisomerases-influencing agents, and (6) chromosomal- and cell cycle metabolism-influencing agents.

3.2.1. Naturally occurring exogenous agents

ES and HeLa cells were exposed to two naturally occurring exogenous agents: γ-radiation and UVC light [1]. Both agents have greatly influenced the evolution of DNA repair since they have been a cause of DNA damage since the beginning of life on earth. However, exposure to ionizing radiation is low from natural exposure but is still a concern primarily due to man made devices and is important for cancer therapy. Ionizing radiation causes a variety of forms of damage, the most toxic being a double-strand break in DNA. Both HRR and NHEJ are required to repair these DNA double-strand breaks. UV light causes single-strand lesions, most often pyrimidine dimers. NER is the major pathway that repairs UV-induced DNA damage.

Survival fractions were determined for cells exposed to naturally occurring exogenous agents

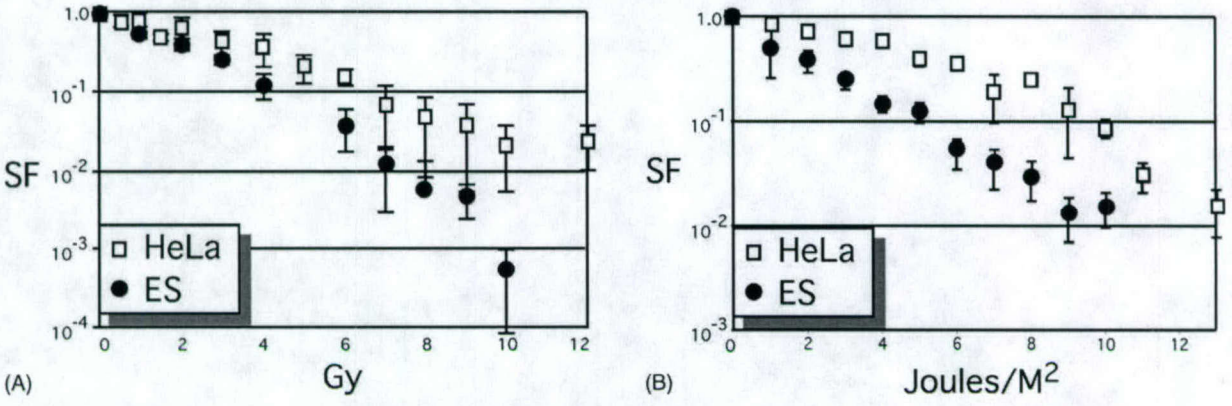

Fig. 1. Dose–response to naturally occurring exogenous agents. The survival fraction (SF), number of exposed cells divided by number of nonexposed cells, is shown on the *Y*-axis. (A) γ-Radiation [measured in Gray (Gy)]. (B) UV light (measured in J/m^2).

(Fig. 1). ES cells are moderately more sensitive to both γ-radiation and UVC compared to HeLa cells. The difference between the dose required to reduce cell number for ES and HeLa cells is moderate with less than a two-fold difference in genotoxin dose required to achieve the same percent reduction in HeLa cells as ES cells throughout much of the curve.

3.2.2. *ROS-altering agents*

ES and HeLa cells were exposed to a variety of agents that impact ROS levels. ROS are natural by-products of metabolism that damage many macromolecules including DNA. A major source of ROS is mitochondrial respiration that produces superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals [1]. Three agents that increase ROS levels are tested: hydrogen peroxide [1], streptonigrin, [19] and paraquat [20]. Two anti-oxidant agents are tested: *N*-acetyl-L-cysteine (NAC) [21] and ebselen [22].

Survival fractions were determined for cells exposed to agents that impact ROS levels (Fig. 2). ES and HeLa cells exhibit about the same sensitivity to streptonigrin, ebselen, and NAC; except that fewer HeLa cells survive higher doses of NAC compared to ES cells. In addition, low-doses of ebselen appear to improve ES cell proliferation since there is a small but consistent increase in cell number (perhaps by reducing oxidative stress). ES cells are moderately more sensitive to paraquat compared to HeLa cells while HeLa cells are moderately more sensitive to H_2O_2 compared to ES cells (less than a two-fold difference in genotoxin dose required to achieve the same percent reduction).

3.2.3. *Alkylating agents*

ES and HeLa cells were exposed to two monofunctional alkylating agents [23]. Monofunctional alkylating agents are electrophilic compounds with affinity for nucleophilic centers in organic molecules and are not only carcinogens but are also intermediates of normal cellular metabolism [1]. These agents have a single reactive group that can interact with a single nucleophilic center in DNA and damage DNA by interacting with ring nitrogens and ring oxygens of bases. These damaged bases are repaired by excision repair pathways: BER, NER and MMR [24–27]. In addition, an alkyltransferase protein transfers the alkyl group from alkylated bases to its own cysteine; thus restoring the base [28]; however, this protein may form a toxic DNA adduct with dibromoethane [29]. Two monofunctional alkylating agents are tested: an SN1 agent, *N*-ethyl-*N*-nitrosourea (ENU) and an SN2 agent, methyl methane sulfonate (MMS). Both agents cause a variety of base modifications; however, the distribution of alkylation damage varies with the agent. Exposure to ENU causes more O^6-alkylguanine and alkylphosphates while exposure to MMS causes more N^7-alkylguanine [1].

Survival fractions were determined for cells exposed to monofunctional alkylating agents (Fig. 3).

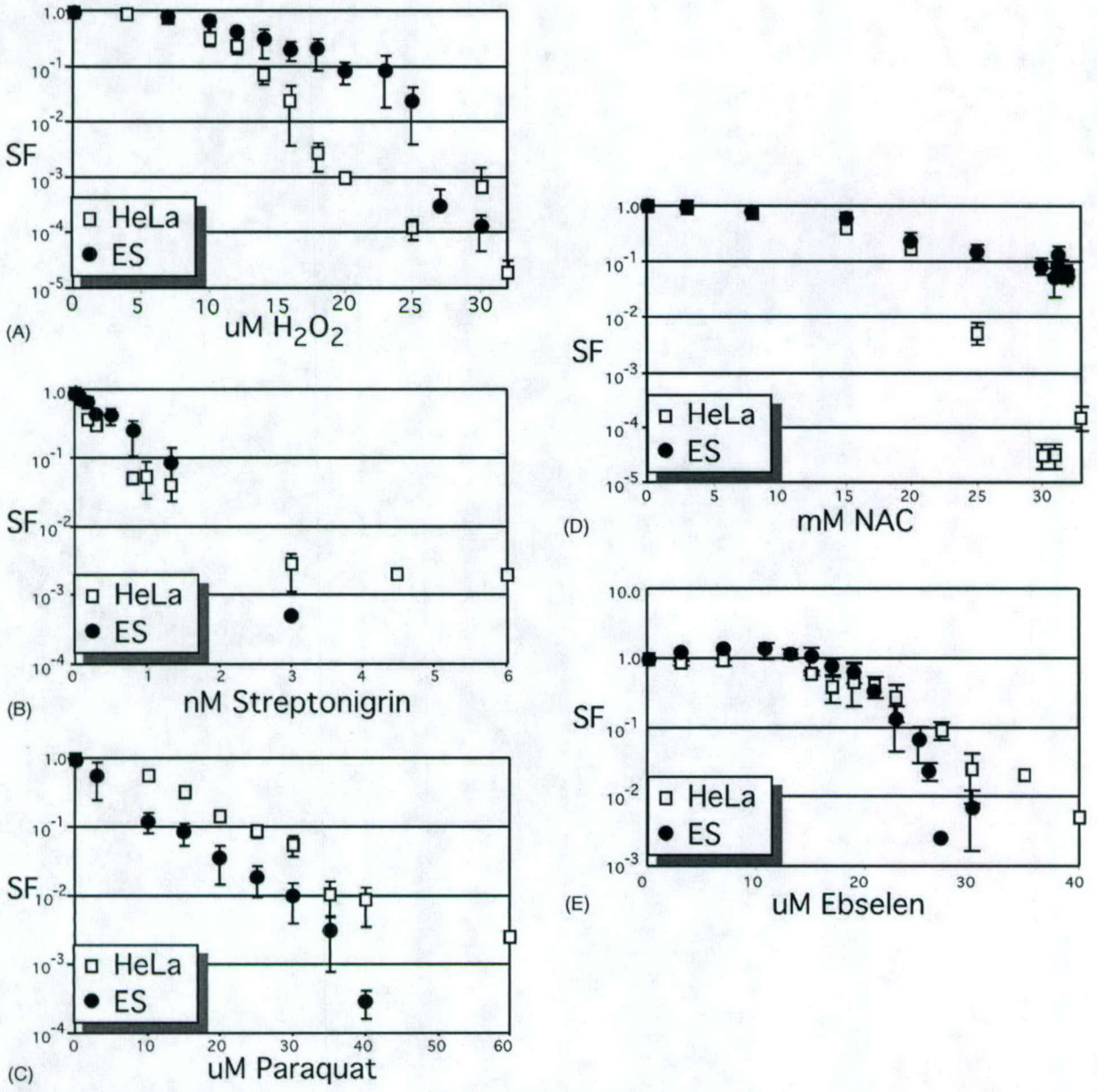

Fig. 2. Dose–response to agents that impact ROS levels. (A) Hydrogen peroxide (H_2O_2). (B) Streptonigrin. (C) Paraquat. (D) *N*-acetyl-L-cysteine (NAC). (E) Ebselen.

HeLa cells are moderately more sensitive to MMS compared to ES cells while ES cells are moderately more sensitive to ENU compared to HeLa cells with about two-fold more agent required to achieve the same percent reduction.

3.2.4. Cross-linking agents

ES and HeLa cells were exposed to two bifunctional alkylating agents that form monoadducts and cross-links. These agents have two reactive groups that can interact with two nucleophilic centers in DNA

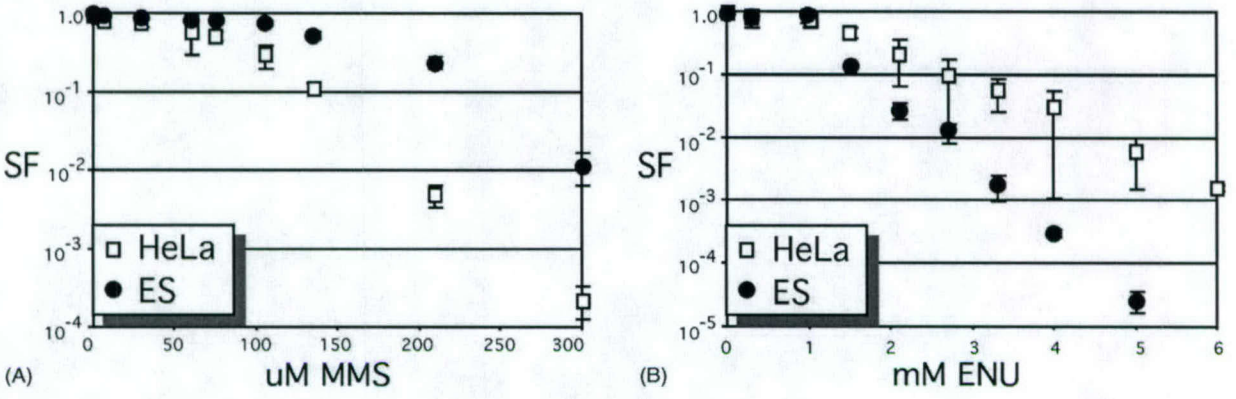

Fig. 3. Dose–response to monofunctional alkylating agents. (A) Methyl methane sulfonate (MMS). (B) *N*-ethyl-*N*-nitrosourea (ENU).

and are thus, able to form monoadducts (interaction of only one reactive group) or intrastrand and interstrand cross-links (interaction of both reactive groups) [1]. Interstrand cross-links prevent DNA replication and transcription since they repress strand separation. DNA double-strand breaks may form when a replication fork encounters an interstrand cross-link [30]. A poorly defined pathway that utilizes components of nucleotide excision repair and homologous recombination repair [31] corrects interstrand cross-links. Two bifunctional alkylating agents are tested: Mitomycin C (MMC) and cisplatin. MMC cross-links by alkylating DNA through covalent linkage with the N2 atom of guanines while cisplatin interacts with the N7 atom of guanine or adenine [32].

Survival fractions were determined for cells exposed to cross-linking agents (Fig. 4). The dose–response curves for ES and HeLa cells are about the same for cisplatin and MMC for reduction of 90% of the cells. However, the remaining 10% of ES cells are more sensitive than HeLa cells to Cisplatin while the remaining 10% of HeLa cells are more sensitive than ES cells to MMC.

3.2.5. *Topoisomerase-influencing agents*

ES and HeLa cells were exposed to agents that impact topoisomerase activity. Topoisomerases are classified into two groups, topoisomerase (topo) I and II and they relax supercoils by generating either single-strand or double-strand breaks, respectively [33–35]. Defective topoisomerase activity may negatively impact a variety of basic cellular functions including DNA replication, transcription and DNA repair; and thus, may impact numerous repair pathways, especially pathways that repair DNA double-strand breaks. Three topoisomerase inhibitors

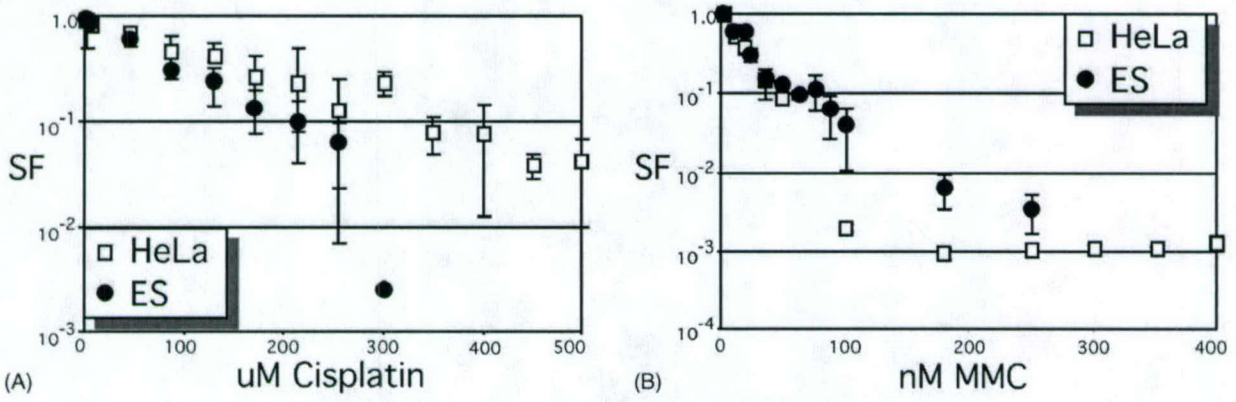

Fig. 4. Dose–response to cross-linking agents. (A) Cisplatin (B) Mitomycin C (MMC).

are tested: camptothecin, etoposide and ICRF-193. Camptothecin is a topo I inhibitor [36] that stabilizes the covalent 3′-phosphotyrosyl intermediate and prevents religation [37–39]. Etoposide is a topoisomerase poison that converts topoisomerase II into a lethal molecule by stabilizing topoisomerase–DNA strand passing reaction intermediates [40,41]. ICRF-193 is a catalytic inhibitor of topo II [42] and perhaps a mild poison [43].

Survival fractions were determined for cells exposed to these topoisomerase inhibitors (Fig. 5). Ninety-nine percent of HeLa cells are more sensitive than ES cells to camptothecin. At many points on the dose–response curve eight-fold or more genotoxin is required to achieve the same percent reduction for ES cells as for HeLa cells. However, ES cells are more sensitive than HeLa cells to both topo II inhibitors, etoposide and ICRF-193. At many points on the dose–response curve 10-fold or more genotoxin is required to achieve the same percent reduction for HeLa cells as for ES cells. In addition, there is a greater than 100-fold difference in the survival fraction that is consistent over the entirety of the dose–response curve.

3.2.6. Chromosomal- and cell cycle metabolism-influencing agents

ES and HeLa cells were exposed to a variety of agents that impact chromosomal and cell cycle metabolism. This is a broad-based classification that tests diverse cellular functions, including DNA replication, chromosomal segregation, and those functions that respond to DNA damage or alterations in chromatin structure. Six agents are tested: hydroxyurea (HU), aphidicolin, 6-thioguanine (6-TG), L-mimosine, colcemid, and trichostatin A (TSA). HU inhibits DNA replication by inhibiting ribonucleotide reductase, which regulates supply of dNTP's [44]. Aphidicolin inhibits DNA polymerases alpha, delta and epsilon [45], producing replication block [46] and single-strand breaks [47]. 6-Thioguanine (6-TG) is a purine anti-metabolite that is metabolized to a toxic base analogue via the purine salvage pathway. L-Mimosine is a potent reversible late G_1 phase blocker of the cell cycle by upregulation of p27/p21 [48,49]; however, the mode of action is complex and may have more than one target in the cell [48]. Colcemid depolymerizes microtubules [50] such that the centrosome fails to duplicate, leading to metaphase arrest [51]. Trichostatin A (TSA) is a histone deacetylase inhibitor that induces hyperacetylated chromatin [52].

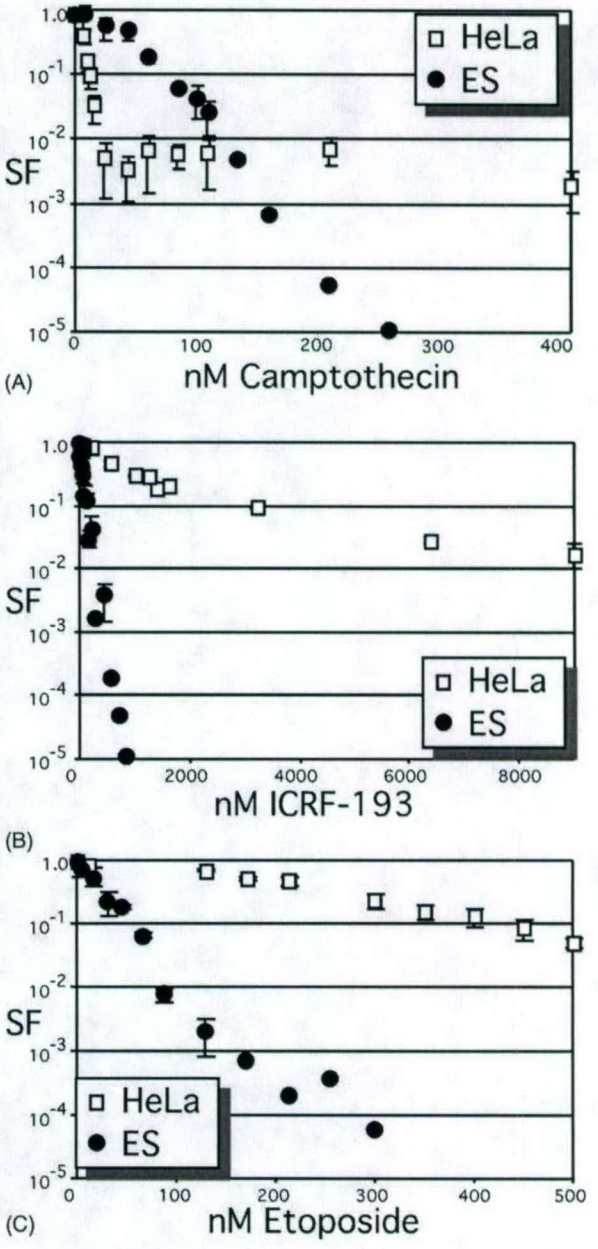

Fig. 5. Dose–response to agents that impact topoisomerase activity. (A) Camptothecin. (B) Etoposide. (C) ICRF-193.

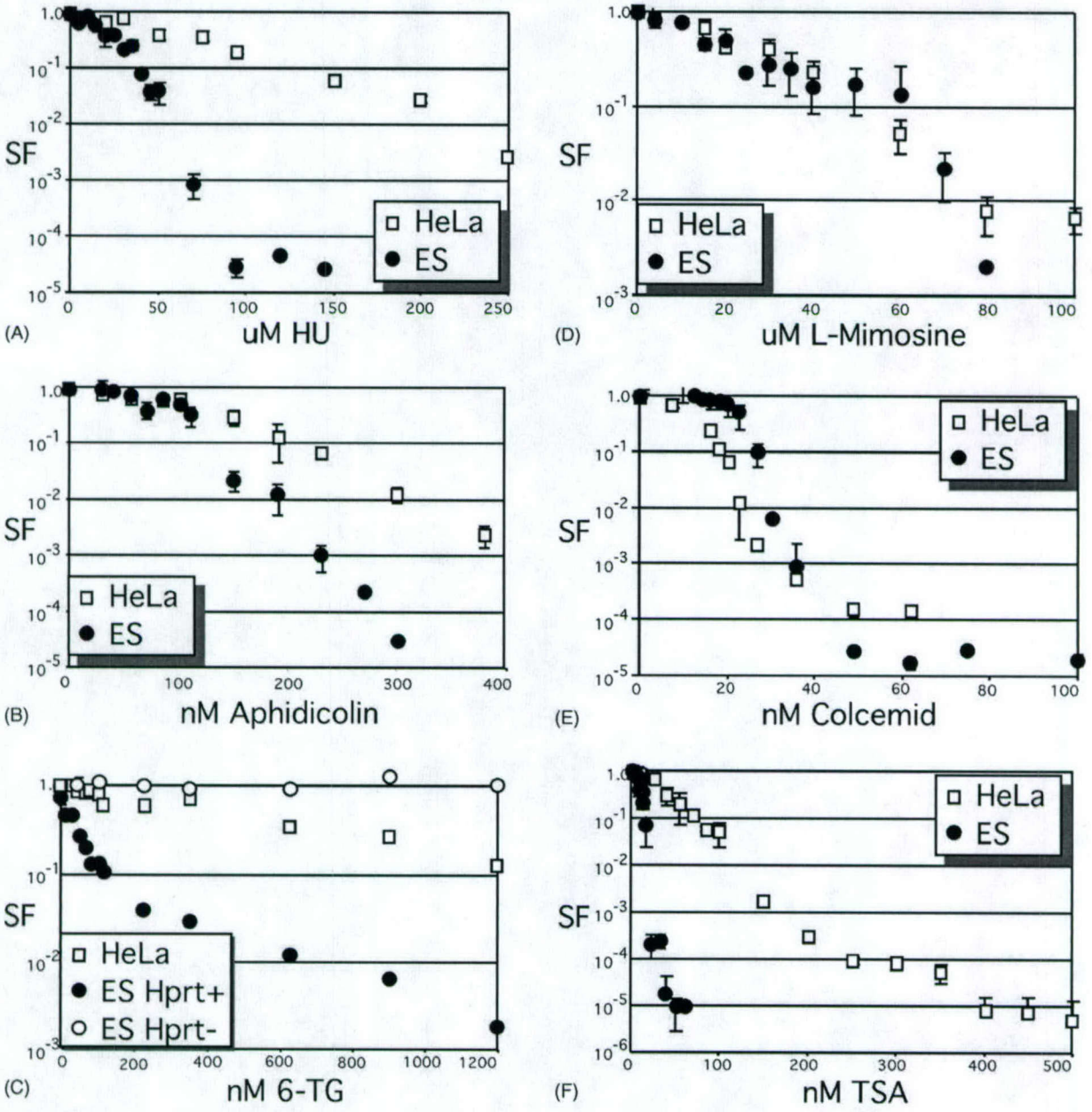

Fig. 6. Dose–response to agents that influence chromatin metabolism and cell cycle responses. (A) Hydroxyurea (HU). (B) Aphidicolin. (C) 6-Thioguanine (6-TG). (D) L-Mimosine. (E) Colcemid. (F) Trichostatin A (TSA).

Survival fractions were determined for cells exposed to these agents (Fig. 6). ES and HeLa cells exhibit about the same sensitivity to L-mimosine and colcemid. However, ES cells are more sensitive to HU, aphidicolin, 6-TG and TSA in that two to six-fold more genotoxin is required to achieve the same per cent reduction in HeLa cells as for ES cells. In addition, there is a greater than 10-fold difference in the SF that is consistent over the entirety of these dose–response curves.

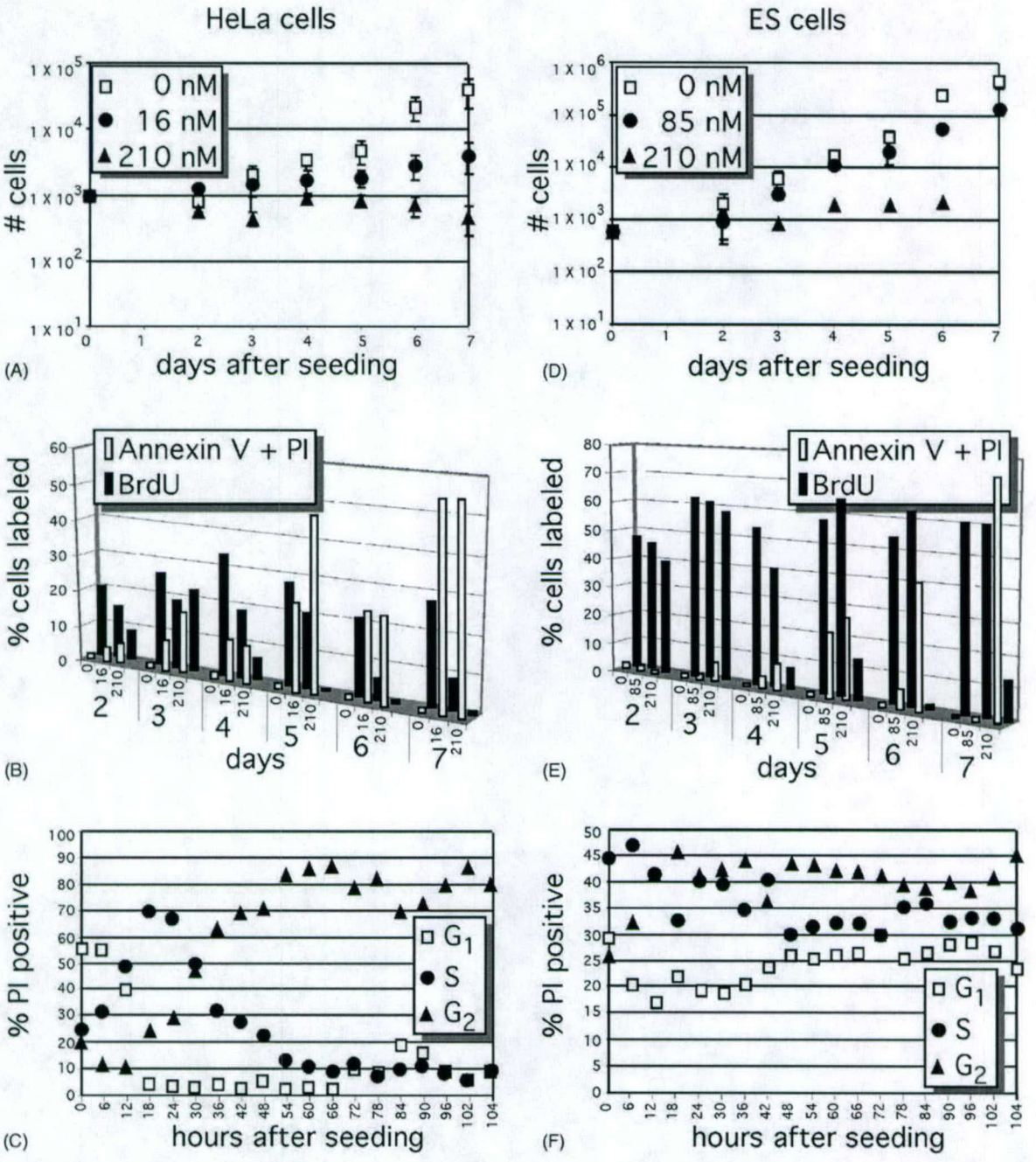

Fig. 7. Time course for cells exposed to camptothecin. HeLa cells were exposed to 16 and 210 nM camptothecin while ES cells were exposed to 85 and 210 nM camptothecin. These cells were seeded on day 0 and camptothecin added on day 1. Cells were observed for every 24 h over 7 days (6 days of exposure to camptothecin). (A) Cellular proliferation of HeLa cells. (B) Analysis of apoptosis (Annexin V) and DNA replication (BrdU) for HeLa cells. (C) DNA content analysis of HeLa cells exposed to 210 nM camptothecin stained with propidium iodide. (D) Cellular proliferation of ES cells after exposure to 85 and 210 nM camptothecin. (E) Analysis of apoptosis (Annexin V) and DNA replication (BrdU) for ES cells. (F) DNA content analysis of ES cells exposed to 210 nM camptothecin stained with propidium iodide.

The function of hypoxanthine phosphoribosyltransferase (Hprt) [53] is a special concern for the 6-TG dose–response curve because HPRT is essential for metabolizing 6-TG into a toxic base. Thus, resistance to 6-TG may be a result of a mutation in *Hprt* (Hprt is on the X chromosome so mutation of only one gene is required to delete function in XY and XO cells). To determine if *Hprt* was mutated, cells resistant to 6-TG were counted and then evenly split onto three plates. The first plate had no selection, the second plate had 10 μM 6-TG (this high concentration has been used to select for Hprt-mutant ES cells) and the third plate had HAT (1 mM sodium hypoxanthine, 4 μM aminopterin, 0.16 mM thymidine, selects for the presence of Hprt). Colonies were counted after 10 days (data not shown). For Hprt + ES cells, colonies grew in media with no selection and in HAT selection but no colonies grew in 6-TG selection; thus these results show that the vast majority of surviving cells, within this dose range, are sensitive to 6-TG and that this assay is testing threshold levels of 6-TG toxicity and not selecting for *Hprt*-mutant cells. However for HeLa cells, about 26% of the number of colonies grew in 6-TG as did in HAT indicating that 10 μM 6-TG is not high enough to stop all colony formation or that about one quarter of the 6-TG resistant cells are mutant for *Hprt*.

3.3. Follow-up to the dose–response curve

The dose–response curve is the first step to determine the concentrations of agent that elicit a reduction in cell number. Once these doses are established, a time course may be performed with a single dose to better understand the dynamics of the cellular response to the agent. A time course is shown after exposure to camptothecin. In addition to cell number, apoptosis and cellular proliferation were measured by staining with Annexin V (recognizes phosphatidyl serine proteins that are exposed to the outer cell membrane in early apoptosis) and by BrdU, respectively. FACS analysis was used for quantitation.

A time course was performed to determine how camptothecin reduces cell number (also done in a 24-well format, supplement 1.1). HeLa cells were exposed to 0, 16 and 210 nM camptothecin and ES cells were exposed to 0, 85 and 210 nM camptothecin (Fig. 7). Cells were seeded on day 0 and camptothecin was added on day 1. Cells were counted every 24 h up to day 7. Cell number was reduced by day 3 (48 h exposure). For HeLa cells, the number of BrdU stained cells declined for cells exposed to either 16 or 210 nM BrdU starting on day 2 compared to unexposed cells. This decline progressed until very few cells stained for BrdU by day 7. The number of Annexin V stained cells increased for cells exposed to either 16 or 210 nM camptothecin starting on day 2 compared to unexposed cells and progressed dramatically until day 7 at which time greater than 50% of the cells were undergoing apoptosis. Similar results were seen for ES cells exposed to 210 nM camptothecin; however, at 85 nM camptothecin, there is no obvious decline in BrdU staining and only a temporary increase in Annexin V staining that peaks at day 5 and then declines, suggesting these ES cells are adapting to this dose. Based on these results, camptothecin reduces cell number by apoptosis for both HeLa and ES cells, although these data do not rule out the possibility that a small fraction of cells may enter senescence.

In association with the time course cells were stained with propidium iodide (PI) and analyzed every 6 h for 104 h for DNA content (done in a 6-well format, see supplement 1.1.3). This analysis shows an increase in the number of cells in G_2 for HeLa cells suggesting that they are unable to progress through mitosis (likely due to breaks that occur during DNA replication). Over this time course the average percent of nonexposed cells were consistent with little variance throughout the cell cycle: G_1, 56.6 ± 4.2; S, 22.7 ± 3.9, and G_2, 20.2 ± 2.2 (not shown). However, there is less change in the distribution of ES cells exposed to camptothecin, demonstrating they are less able to inhibit cell cycle progression in response to DNA damage [54].

4. Discussion

Here we present a genotoxic profile for ES cells and HeLa cells by measuring the dose–response to 20 genotoxins; this screen has advantages over other screens. (1) Most investigators are biased and tend to focus on specific lesions corrected by only a subset of pathways. This broad-based screen will minimize investigator bias and may elucidate an unsuspected function for the altered gene product. (2) A single person utilizing standard tissue culture equipment can

perform this screen; therefore, it can be performed by small to average-size labs that do not have the resources to conduct large-scale screens. (3) Assays for 19 of 20 genotoxins observe cell proliferation and do not rely on colony formation; thus, cells that poorly form colonies can be tested. (4) This screen is sensitive since it measures cell proliferation over about one week while the cells exhibit linear growth on a log scale. Therefore, reduction in cell number by either cell cycle checkpoints, apoptosis or senescence will be observed if these cellular end points occur at any time during that week, even if these cellular responses are only evident for a brief period. This is especially important for agents that take several days to produce a toxic level of damage or if cells adapt to the damage. For example, ES cells exposed to 85 nM camptothecin show only mild levels of apoptosis that was observed on days 3 and 4 (<10%) and only a mild reduction in BrdU incorporation observed on day 4 (23%). Yet there is clearly a reduction in cell number by day 5 that can still be measured by day 7. This would have been missed for assays that expose cells to genotoxin for only several hours and measure only apoptosis 24–48 h later (these assays do not record reduction in proliferation due to checkpoints or senescence). Additionally, the longer length of exposure may reveal data more relevant for testing cancer cells since exposure to chemotherapeutics occurs over days, not hours. (5) This dose–response screen is readily adaptable to a time course that can be used to quantitate both proliferation and apoptosis by FACS analysis. Other markers of interest may be observed to determine cell fate in response to genotoxin.

4.1. Comparison of dose–response curves

Even though a meaningful direct comparison cannot be made between individual dose–response curves for ES and HeLa cells, due to their different proliferation rates and different chromosomal compositions (ES cells are euploid while HeLa cells are aneuploid), an indirect comparison can be made between all dose–response curves. For this indirect comparison, dose–response curves can be placed into three groups. Group 1: ES cells and HeLa cells exhibit relatively the same level of sensitivity to genotoxin for the majority of the population (streptonigrin, ebselen, cisplatin MMC, L-mimosine, colcemid). Group 2: ES cells exhibit more sensitivity than HeLa cells to genotoxin (HU, Aphidicolin, 6-TG, TSA, etoposide, ICRF, ENU, radiation, UVC, paraquat). Group 3: HeLa cells exhibit more sensitivity than ES cells to genotoxin (NAC, H_2O_2, MMS, camptothecin).

The most striking observation is that ES cells, compared to HeLa cells, are much more sensitive to agents that interfere with S phase relative to other agents. These agents include HU and aphidicolin that directly interfere with DNA replication. In addition there is 6-TG, an anti-metabolite that interferes with replication through the mismatch repair pathway [55,56] and there are both topoisomerase II inhibitors (etoposide and ICRF-193) that interfere with the replication fork. Agents that impair S phase may impact ES cells more than HeLa cells due to their different proliferation rates (ES cells proliferate about 2.3× faster than HeLa cells). However, ES cells are more resistant than HeLa cells to camptothecin, whose action occurs during S phase. Therefore, disruption of S phase alone cannot fully explain the general observation that ES cells are more sensitive to agents that interrupt S phase.

By comparison, there is less difference between ES and HeLa cell survival for agents that directly damage DNA or impact ROS levels; genotoxic agents shown in Figs. 1–4 are placed into all three groups. The difference between these SF curves is not that striking for most of these agents. These data suggest that ES cells and HeLa cells possess a similar capacity for repairing DNA damage that is not directly associated with replication. In addition, there is little difference in their ability to survive colcemid, suggesting their capacity to respond to microtubule depolymerization is about the same.

There are some agents that show a bi-modal impact such that one cell type is more sensitive than the other at low doses but the reverse is true at high doses. However, the part of the bi-modal curve, at high dose, represents only a very small fraction of cells. A good example is the topoisomerase 1 inhibitor, camptothecin. The survival fraction curve for ES cells exposed to camptothecin is typical (more or less a linear line); however, the survival fraction curve for HeLa cells shows a sharp decline over a small dose-increase and then a leveling off over a large dose-increase (the latter part of this curves represents less than 1% of the population). The majority of HeLa cells undergo apoptosis after exposure to camptothecin since they stain

positive for Annexin V. However, the small fraction of HeLa cells that appear resistant to camptothecin may enter senescence. Since HeLa cells are aneuploid, these bi-modal curves may be selecting for a small population of cells that have an advantage for survival (per haps deficient in apoptosis).

4.2. Relevance to cancer therapy

A database with genotoxic profiles of cancer-derived cells may be helpful with diagnostics, prognostics and developing therapeutic protocols. The ontogeny of cancer is due to a progression of genetic alterations that change basic cellular metabolism and as a consequence may influence the performance of anti-cancer therapeutics. Thus, anti-cancer therapy protocols must be individualized for each tumor and development of these protocols may be costly with regard to both money and time. Since many anti-cancer agents function by damaging DNA [57] or by inhibiting basic DNA metabolic pathways, a genotoxic profile database could be integrated with expression profiles and CHG databases to quickly understand the cancer and enable development of a therapeutic regiment.

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The impact of DNA damage, genetic mutation and cellular responses on cancer prevention, longevity and aging: observations in humans and mice

Paul Hasty*

Department of Molecular Medicine, University of Texas, Health Science Center at San Antonio, 15355 Lambda Drive, San Antonio, TX 78245, USA

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Abstract

Over the past 5 years, data collected from the mouse suggest that pathways important for either preventing or resolving DNA damage are longevity assurance mechanisms whose critical overall function is somatic cell maintenance, a necessary part of cancer prevention. These pathways include those that reduce DNA damage levels caused by exogenous sources, replication errors and by-products of cellular respiration. Unresolved DNA damage leads to permanent mutations in the genetic code that may be oncogenic. Therefore, pathways that resolve DNA damage are important anti-cancer mechanisms. As an important line of defense, there are a variety of pathways that repair DNA damage. In addition, there are anti-cancer pathways that respond to DNA damage by either preventing cellular replication or inducing cell death. Genes in these pathways, termed longevity assurance genes (LAG), code for proteins that reduce cancer incidence and as a result assures a sufficiently long health span needed for reproduction. Data from mouse models, many that were originally designed to study cancer, are showing that a potential consequence of DNA damage and responses to DNA damage is aging; these models support the hypothesis that at least some aspects of normal aging are the consequence of anticancer mechanisms designed to deal with damaged DNA.

Keywords: DNA damage; Aging; Mice

The free radical theory of aging, originally proposed by Harman (1956), states that age-related decline is due to an accumulation of damage to macromolecules caused by the by-products of oxidative respiration, called reactive oxygen species (ROS). ROS damage a variety of macromolecules including DNA. DNA may also be damaged by other agents and by errors in replication. Damaged DNA is particularly harmful since it may be fixed into a permanent mutation and passed onto daughter cells. Decreased or aberrant cell function may result from these mutations. Thus, genomic maintenance is essential to maintain both cellular and organismal homeostasis. A variety of pathways are present to maintain the soma at a cellular level by addressing DNA damage caused by genotoxic agents and replication errors. These pathways perform functions important for stress resistance and include pathways that reduce ROS, repair DNA damage, halt the cell cycle or induce cell death. These functions are important for longevity by preventing pathology associated with the accumulation of DNA damage and mutations, most notably cancer. Recent data in the mouse suggest that the process of aging may be due to, at least in part, the interplay between those forces that damage DNA and those forces designed to deal with that damage. Basically life span and onset of aging is directly related to the quality of soma maintenance.

1. DNA damage and mutations

A wide range of agents that originate from both exogenous and endogenous sources may damage our

* Tel.: +1 210 567 7278; fax: +1 210 567 7247.
E-mail address: hastye@uthscsa.edu.

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genetic material (Friedberg et al., 1995; Marple et al., 2004). Exogenous sources include UV light, ionizing radiation and some chemical agents. Endogenous sources include by-products of cellular metabolism, most notably, ROS that are derived from oxidative respiration and lipid peroxidation. In addition, DNA can be damaged by spontaneously arising abasic sites, errors in replication and errors in repair. As a result the integrity of our genetic material is constantly being challenged and this challenge threatens normal cellular function.

Increasing evidence demonstrates that DNA damage accumulates with age in mice. For example unrepaired DNA double-strand breaks accumulate in a variety of tissues as mice age (Sedelnikova et al., 2004). In addition, the levels of DNA damage correlate with husbandry conditions that alter life span. For example, mice fed ad libitum have a shorter life span and earlier onset of aging phenotypes compared to mice fed a limited diet (caloric restriction). Both groups of mice exhibit increased levels of 8-oxo-2-deoxyguanosine (oxo8dG) with age in multiple tissues; however, this increase is seen sooner in the ad libitum fed mice than in the calorie-restricted mice (Hamilton et al., 2001). Thus, the levels of DNA damage correlate directly with biological age.

Age-related phenotypes may be the result of increased genetic damage. For example, with age there is a cognitive decline in the human brain. Recent data suggest reduced expression in a variety of genes with age in the frontal cortex that include those important for stress response, antioxidant defense and DNA repair (Lu et al., 2004). Furthermore, reduced repair of small single-strand lesions (a defect in base excision repair) likely resulted in an accumulation of DNA damage that was selective for the promoters of some of these under-expressed genes. Thus, unrepaired DNA damage may contribute to age-related cognitive decline in humans.

Since DNA damage accumulates with age, one would predict the same to be true for mutations that often result from erroneous repair. In fact, this has been shown to be true. There is an age-related increase in mutations that is tissue specific in mice (Dolle et al., 2000; Vijg and Dolle, 2002). These different mutational fingerprints indicate that some DNA metabolic pathways are more important for certain cell types and tissues than for others; this is understandable since tissues are composed of cells with different mitotic activities, metabolic rates, and exposure to different toxins. Interestingly, in human and mouse lymphocytes the frequency of chromosomal aberrations (Ramsey et al., 1995; Tucker et al., 1999), as well as mutations at the HPRT locus (Jones et al., 1995; Dempsey et al., 1993), increases with age at about the same rate, as a function of their life span rather than chronological time. This suggests that the accumulation of mutations with age is related to the rate of aging and could be a function of the repair phenotype of the species (Hart and Setlow, 1974). Indeed, mutation accumulation at HPRT has been found to accelerate in a mouse model of premature aging (Odagiri et al., 1998) and decelerate in calorically restricted mice (Dempsey et al., 1993). Thus, DNA damage and mutations increase with biological age.

Accumulation of genetic mutations result in age-related phenotypes, especially cancer. Cancer is an age-related disease that is caused by a variety of factors, including accumulation of genetic mutations (DePinho, 2000). For a single tumor cell, there may be hundreds of genetic aberrations; either changes in the genetic sequence, copy number or expression, such that a hallmark of cancer is genomic instability (Balmain et al., 2003).

2. DNA repair

For humans, dysfunction in specific DNA repair pathways has proven to predispose an individual to specific tumors (Hoeijmakers, 2001). For example, a mutation in a gene important for mismatch repair (MMR) predisposes an individual to colon cancer. MMR corrects base pair mismatches arising from replication slippage at dinucleotide repeats. In addition, MMR corrects other types of damage to single strands of DNA. A mutation in either Brca1 or Brca2 predisposes women to mostly breast cancer. Both proteins repair double-strand breaks in DNA by homologous recombination (HR). A mutation in a gene important for nucleotide excision repair (NER) predisposes an individual to skin cancer (de Laat et al., 1999). NER repairs bulky lesions to a single-strand of DNA caused by a variety of agents including ultraviolet light. Thus, decreased activity of a pathway designed to repair a specific subset of lesions predisposes the individual to a subset of tumors that may be observed in the entire population.

These segmental tumor spectra, likely reflect tissue and cellular specificity of the dysfunctional DNA repair pathway. As one could imagine, certain cell types would have a greater need for specific DNA repair pathways than others due to a variety of factors including metabolic rate, mitotic index, differentiation status and level of exposure to specific genotoxins. For example NER prevents cancer for tissues exposed to environmental genotoxins like ultraviolet light and food components. Therefore, as shown in humans and mouse models, impaired NER results in skin and gastrointestinal cancers, since both are caused by exposure to exogenous agents: sunlight and food, respectively.

Deficits in DNA repair predispose individuals to age-related phenotypes in addition to cancer. These phenotypes are described as segmental progeroid syndromes, since only a subset of the age-related pathologies that are normally observed in the entire population are displayed. This is similar to DNA repair deficiencies that predispose an individual to a segmental cancer spectrum. A complete description of these syndromes and their phenotypes has been provided (Martin, 1978). These syndromes were

described based on clinical observations; therefore, it is striking that most of them have been found to result from defective chromosomal metabolism. Even for those exceptions (Hutchinson–Gilford's and Down's syndrome) evidence is mounting that the root cause of premature aging is chromosomal dysfunction. For Hutchinson–Gilford (due to a defect in lamin A) there are changes in nuclear structure that may impact regulation of gene expression and DNA replication (Goldman et al., 2004). For Down's syndrome there could be an exaggerated mitotic checkpoint as described in more detail later in this review. Thus, chromosomal dysfunction appears to predispose an individual to certain age-related pathologies.

The best-known segmental progeroid syndrome is Werner's syndrome (WS) [Goto, 1997 #158; Martin, 1999 #159]. An inactivating mutation in *WRN*, a homolog of the *E. coli RECQ* gene, causes WS [Yu, 1996 #198]. WRN is likely important for replication, repair and transcription. Cells deficient in WRN exhibit genetic instability that includes large chromosomal deletions. Individuals with WS develop, two to three decades prematurely, atrophic skin, thin gray hair, osteoporosis, type II diabetes, cataracts, arteriosclerosis, and cancer [Goto, 1997 #158; Martin, 1999 #159]. Interestingly, about half the cancers are mesenchymal in origin, in contrast to cancers that develop in normal individuals, of which 90% are epithelial in origin [Goto, 1997 #158; Martin, 1999 #159]. WS individuals typically die in the fifth decade of life, primarily of cardiovascular disease or cancer. There are five *RECQ*-like genes in mammals. All encode $3'\rightarrow5'$ DNA helicases, and at least three, WRN, RTS (Rothmund Thomson's syndrome) and BLM (Bloom's syndrome gene) are associated with premature aging and/or cancer prone syndromes in humans [van Brabant, 2000 #928; Mohaghegh, 2001 #779].

Mice genetically altered for DNA repair pathways have been made in an attempt to better understand cancer predisposition in humans and one outcome of these studies is that some DNA repair deficits predispose an individual to age-related pathologies that are of greater scope than just cancer. Defects in a variety of DNA repair pathways may cause these age-related phenotypes. These genetic mutations and their affected pathways have been reviewed (Hasty et al., 2003; Warner and Sierra, 2003) and will not be discussed in detail here. Briefly, the DNA repair pathways include those that repair double-stranded lesions: homologous recombination (HR), nonhomologous end joining (NHEJ) and cross-link repair (CLR). Defects in specific genes that result in premature aging include Brca1 for HR; Ku80 and DNA-PK_{CS} (Espejel et al., 2004) for NHEJ, and Ercc1 for CLR. Furthermore, the cell likely views defective telomeres as unrepaired DNA double-strand breaks and mice with defective telomeres exhibit an early onset of aging pathologies. Progeroid syndromes have also been reported for the repair of a single-strand lesion encountered during transcription [transcription coupled repair (TCR)]. Most notable is a subtle defect in Xpd, which is one of the helicases for transcription factor IIH (TFIIH). In addition to damage in nuclear DNA, damage to mitochondrial DNA may also affect aging. Recently a mouse model was described that expresses a defective polymerase for mitochondrial DNA (Trifunovic et al., 2004). This polymerase was disabled for its proofreading capability and these mice exhibited instability of the mitochondrial DNA along with a variety of age-related pathologies similar to mice defective in the repair of nuclear DNA. Thus, a defect in a single gene in one of multiple DNA repair pathways may cause a similar pleiotropic aging phenotype.

It is curious that only a small percentage of DNA-repair deficient mice exhibit an aging phenotype (Friedberg and Meira, 2003). There are several explanations for this. First, many of the premature aging mice are deficient for a protein with broad function. That is the protein repairs or responds to a broad spectrum of DNA lesions. Second, DNA repair defects often times cause early death (Friedberg and Meira, 2003) and as a result prohibits observation of subtle late-onset pathologies. A good example for both points is the trichothiodystrophy phenotype in mice caused by a subtle mutation in Xpd (de Boer et al., 2002). Xpd has broad function since it is one of the helicases in TFIIH and therefore impacts both DNA repair and transcription. Xpd is also essential for cell viability and its deletion results in early embryonic lethality. Therefore, the aging phenotype was only realized by studying a subtle mutation that only weakened function. Third, the full range of phenotypes may not be realized due to complicated genetic interactions. A good example is WRN. For humans deletion of WRN results in an obvious aging phenotype, but in mice there is almost no observable phenotype. However, the human WS phenotype is observed in mice when these mice have shortened telomeres (Chang et al., 2004; Du et al., 2004). This could simply be due to the fact that laboratory strains of mice possess much longer telomeres than humans. Thus, specific genetic backgrounds are often required to reveal aging phenotypes.

It is also curious that similar aging phenotypes are observed for genetically altered mice even when different DNA repair pathways are affected by the alteration. Although the aging phenotypes are described as segmental, they commonly exhibit a wide range of age-related characteristics that affect a diverse set of cell-types and tissues. These phenotypes have been reviewed (Hasty et al., 2003; Hasty and Vijg, 2004) and will not be discussed here in detail. Briefly, some of the commonly observed pathologies are osteopenia, skin atrophy, graying fur, alopecia, skin ulcers and hepatocellular inclusions. Also common are defects in wound repair, and cells derived from these mice often exhibit replicative senescence and genomic instability. All these pathologies are observed for control mice only at late age; thus, they reflect normal aspects of aging. So it is intriguing that similar age-related phenotypes can be caused by deficits in diverse DNA repair pathways that increase different forms of DNA damage.

3. Cell cycle checkpoint and apoptosis

Even though deficits in diverse DNA repair pathways result in an increase of various types of DNA damage, cellular responses to these different types of damage have a similar outcome; that is, they may induce either cellular senescence or apoptosis. Both cellular responses are designed to prevent cancer-causing mutations but may also, as an indirect consequence, cause certain aspects of aging (Campisi, 2000; Bree et al., 2002; Pelicci, 2004). Replicative senescence refers to the limited proliferative potential (replicative life span) and eventual arrest exhibited by normal cells in tissue culture (Hayflick, 1965), while apoptosis is programmed cell death that occurs normally during development and in response to DNA damage (Reed, 1999). Either replicative senescence or apoptosis may be induced by responses to ROS-induced DNA damage (Parrinello et al., 2003) that would otherwise be permanently fixed to a mutation (Busuttil et al., 2003). Mutations give rise to cancer; thus replicative senescence and apoptosis are important anti-cancer mechanisms that could explain the similarity between the aging phenotypes exhibited by mice deficient in diverse DNA repair pathways. Support for this hypothesis comes from a recently described mouse model suggesting that the spindle assembly checkpoint protein, BubR1, is a longevity assurance gene that impacts aging phenotypes (Baker et al., 2004). Mice deleted for BubR1 die early during development (Wang et al., 2004); however, expression of a hypomorphic allele results in reduced life span and an early onset of aging features similar to some DNA repair deficient mice. There was marked genomic instability due to deficient chromosomal segregation (not DNA damage), yet only few mutant mice exhibited cancer, suggestive of increased cellular responses. Decreased cancer incidence is also observed in some of the DNA repair mutant mice that exhibit aging phenotypes, also suggestive of increased responses to poorly repaired DNA damage. In addition, Down's syndrome in humans has been described as a segmental progeroid syndrome but the cause of the early aging phenotypes is uncertain and does not appear to be based on deficits in DNA repair; however, the BubR1 hypomorphic mice suggest that aging in Down's syndrome people may be due to an increased mitotic checkpoint since the genetic defect that causes Down's syndrome is trisomy for chromosome 21 (Fernandez-Capetillo and Nussenzweig, 2004).

Further support for the hypothesis that cellular responses to DNA damage cause some aspects of aging comes from analysis of p53, a tumor suppressor gene. p53 induces apoptosis and cell cycle checkpoints in response to conditions that negatively impact successful DNA replication (Ko and Prives, 1996). Removing p53 increases replicative potential (Harvey et al., 1993) while overexpression decreases replicative potential and promotes replicative senescence (Sugrue et al., 1997). Thus, conditions that initiate the p53-dependent G_1/S checkpoint, like DNA damage, induce cell cycle arrest in G_1 that may ultimately result in replicative senescence, especially if the DNA damage persists. Therefore, p53 activity may be increased in DNA repair deficient people and mice that exhibit precocious aging. To support this notion, replicative senescence of fibroblasts derived from mice with premature aging is dependent on p53 (Chin et al., 1999; Difilippantonio et al., 2000; Lim et al., 2000).

The role p53 dosage plays in aging for DNA repair deficient mice are most clearly shown for Brca1-mutant mice, because their aging phenotype is only observed in a p53-heterozygous mutant background (Cao et al., 2003). In a p53 wild type background the Brca1-defective mice die early during embryogenesis while in a p53-homozygous mutant background these mice exhibit a high cancer incidence (Xu et al., 2001). Thus, p53 levels are critical for observing both the aging and cancer phenotypes for Brca1-deficient mice.

Investigation of two independently derived mouse models that overexpress a short isoform of p53 that lacks some of the N-terminus, further suggests that some aspects of aging are the consequence of cellular responses (Tyner et al., 2002; Maier et al., 2004). Complete deletion of p53 function enhances tumor susceptibility and reduces longevity in the mouse (Donehower et al., 1992). However, a truncated version of p53 causes increased cancer resistance, yet decreased longevity accompanied by the accelerated onset of a variety of aging phenotypes that are similar to some DNA repair deficient mice. The short p53 isoform, called p44 in the mouse, requires full-length p53 to cause this aging phenotype. It is likely that p44 is a naturally occurring protein since it was found in tissue extracts from nontransgenic mice (Maier et al., 2004) and human cells (Courtois et al., 2002; Yin et al., 2002). The short isoform stabilizes p53 in the presence of Mdm2 and alters the expression levels of p53-induced gene products (Yin et al., 2002). Thus, overexpression of the p53 isoform likely increases some aspects of p53 function that reduces tissue regenerative responses which is consistent with an accelerated loss of stem cell functional capacity (Dumble et al., 2004). Therefore, these mouse models show that activation of p53 by a dominant-allele accelerates the aging process.

4. Longevity models

Transgenic mice that overexpress p44 exhibit hyperactivation of the insulin-like growth factor (IGF) signaling axis (Maier et al., 2004); a pathway known to negatively regulate longevity by inhibiting a Forkhead transcription factor in both nematodes and flies (Guarente and Kenyon, 2000). Many of these long-lived mutants are resistant to stress, including oxidative stress (Johnson et al., 2001). The IGF signaling pathway likely does the same in mammals since reduction of the IGF-1 receptor improved resistance to

genotoxic agents and extends life span in mice (Holzenberger et al., 2003). Additional evidence that ROS-induced damage contributes to aging comes from studies of the 66 kDa isoform of SHC ($p66^{SHC}$) that transduces extracellular signals, including those generated by growth factors and stress. Importantly, $p66^{SHC}$ knockout mice had improved resistant to oxidant (H_2O_2)-induced apoptosis and exhibited a significantly increased lifespan compared to wild-type mice (Migliaccio et al., 1999). Moreover, $p66^{SHC}$-generated ROS decreases the activity of a Forkhead transcription factor, FOXO3a (a.k.a. FKHRL1) (Nemoto and Finkel, 2002), which, in turn, stimulates DNA repair. Therefore, mouse models that extend life span and delay aging are resistant to oxidative stress, just the opposite of those DNA-repair deficient models of accelerated aging.

5. Conclusion

Observations in mouse models with altered ability to resolve ROS-induced DNA damage often reveal a direct correlation between cancer incidence, aging and longevity (Fig. 1). Improved resistance to agents that damage DNA increases the latency of aging and extends life span while reduced resistance to DNA damage does the opposite as shown by mouse models and some human syndromes. Impairment of a DNA repair pathway may either increase cancer-causing mutations or alternatively increase cellular responses that in turn accelerates aging. Therefore, mouse models appear to show that levels of DNA damage have consequences for both cancer and aging and that at least a part of aging is due to cellular responses to DNA damage that are designed to prevent cancer. If this is true, it means that aging is important for both increased life span (cancer prevention early in life) and decreased life span (age-related deterioration late in life). This concept is referred to as antagonistic pleiotropy (genetic alterations that initially increase fitness but later decrease fitness) (Williams, 1957). By this reasoning, aging is important for extending life span early in life by reducing cancer incidence and would likely be important for allowing time for reproduction. Many cancer models support this concept; for example, p53-mutant mice exhibit cancer so early that the females frequently are unable to birth and wean more than one litter. Other cancer models are similar. Based on the potential relationship between anti-cancer mechanisms and aging, one might predict that therapeutic intervention to ameliorate aging would be oncogenic and therefore, counterproductive. This would be true if such therapeutic interventions directly reduced cell cycle checkpoints or apoptosis; however, the IGF-1 receptor and $p66^{SHC}$ mutant mouse models demonstrate that amelioration of aging is possible without increasing cancer risk. Agents that decrease either the IGF-1 or $p66^{SHC}$ pathways may improve resistance to oxidative damage and perhaps ameliorate some of the negative consequences of aging without increasing cancer risk. The multiple accelerated aging mouse models suggest therapeutics may target organs like skin and bone since all of these models exhibit signs of early aging in these organs. These mouse models would also be suitable for initial tests of therapeutic efficacy and potential cancer risk. Thus, mouse models of aging and longevity suggest that therapeutics for geriatric therapy may address aging mechanisms by improving stress resistance.

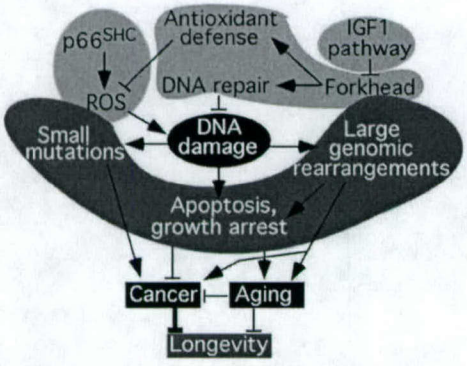

Fig. 1. DNA damage as the central force for both cancer and aging. Agents, like ROS and environmental toxins, cause DNA damage that may become a permanent mutation. Such mutations may increase cancer risk. A variety of pathways are present to prevent cancer-causing mutations: antioxidant defense reduces ROS, DNA repair corrects DNA damage and if that fails, then apoptosis or growth arrest prevents proliferation of mutated cells. These responses, designed to prevent cancer, could also be a causal factor for aging. Both cancer and aging decrease life span, but cancer more so than aging since it may occur earlier in life, especially with a defect in any longevity assurance mechanism. Light blue, pathways that improve longevity. Light red, pathways or agents that impair longevity. Dark red, cellular events that impair longevity (note, apoptosis and growth arrest may have dual consequences since they reduce cancer risk but also may promote aging). Black, the central event of DNA damage and if not repaired correctly, its potential outcomes of cancer and aging. Dark blue, longevity that is promoted by longevity assurance genes.

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